

THE GENETIC SUSCEPTIBILITY/RESISTANCE TO FLUOROSIS  
AMONG DIFFERENT INBRED MOUSE STRAINS

by

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## INTRODUCTION

Fluoridation of community drinking water to prevent dental caries is considered to be one of the 10 most important public health achievements of the 20<sup>th</sup> century.<sup>1</sup> Concurrent with the decline in dental caries is an increase in the prevalence of dental fluorosis, a side effect of systemic exposure to greater than optimal levels of fluoride. The prevalence of dental fluorosis in recent years has been estimated to be between 7.7 percent and 80.9 percent of the population in communities with fluoridated water and from 2.9 percent to 42 percent in communities with nonfluoridated water.<sup>2-4</sup> Although the increase in prevalence has occurred primarily in very mild forms of dental fluorosis, there is evidence that the prevalence of moderate and severe fluorosis has increased as well.<sup>2</sup> In addition to those major risk factors previously identified (use of fluoridated drinking water, fluoride supplements, fluoride toothpaste, and infant formulas),<sup>3</sup> other factors including critical periods of fluoride exposure during tooth development and individual variation are important as well.

While it is well-accepted that fluoride interacts with mineralized tissues and at elevated concentrations disturbs the mineralization process,<sup>5</sup> the molecular mechanisms that underlie the pathogenesis of dental fluorosis are not known. We hypothesized that an individual's genotype could influence susceptibility or resistance to developing dental fluorosis. We tested this hypothesis using a mouse model system where continuous eruption of the mouse incisor over a relatively short period of time could be monitored,



and where genotype, age, gender, food, housing, and drinking water fluoride level could be rigorously controlled.

The purpose of this study was to determine if there are differences in fluoride sensitivity or resistance among different inbred mouse strains. Numerous earlier studies support the mouse as a useful model in determining the effects of fluoride on dental and skeletal development.<sup>6-14</sup> Interestingly, those studies were not specifically designed to address questions related to genetic sensitivity/resistance to environmental fluoride. Because mice like other rodents continue to grow their upper and lower incisors, the effects of fluoride on ongoing amelogenesis can be monitored.

## REVIEW OF LITERATURE

Dental caries (i.e. tooth decay) is an infectious, multifactorial disease affecting most persons in industrialized countries and some developing countries.<sup>15</sup> Widespread use of fluoride has been a major factor in the decline in the prevalence and severity of dental caries in the US and other economically developed countries. When used appropriately, fluoride is both safe and effective in preventing and controlling dental caries.<sup>15</sup> The safety and efficacy of fluoride use to prevent dental caries has been extensively investigated. Epidemiological studies began with the search for the cause of “Colorado Brown Staining” and led to the first community fluoridation program instituted in 1945 in Grand Rapids, MI.<sup>16</sup> At that time, public health officials assumed that drinking water would be the major source of fluoride for most US residents.<sup>15</sup>

Fluoridation of community water supplies for the prevention of dental caries remains one of the top 10 public health interventions of the last century.<sup>1,16</sup> In the 1930s, it was found that the ingestion of excessive quantities of fluoride during the period of pre-eruptive tooth formation resulted in chronic endemic fluorosis.<sup>17</sup> Studies in the 1940s showed a decline in caries that was attributable to fluoride present in the drinking water.<sup>18</sup> It has been observed that maximum protection against dental caries is received when the water supply contains 1 ppm (part per million) of fluoride. It has also been demonstrated that when the concentration of fluoride in the water supply is 1 ppm, fluorosis affected less than 10 percent of the children examined.<sup>19,20</sup>



Concurrent with the decline in dental caries is an increase in the prevalence (the number of cases at a given time) of dental fluorosis, a side effect of fluoride exposure. In recent years, there has been growing evidence that the prevalence of dental fluorosis is increasing in both optimally and negligibly fluoridated communities.<sup>2,21-30</sup> The prevalence of dental fluorosis in recent years ranged between 7.7 percent and 80.9 percent of the population in communities with fluoridated water and from 2.9 percent and 42 percent in communities with nonfluoridated water.<sup>2,3,31</sup> All the data indicate that the current prevalence of dental fluorosis is substantially greater than that observed by Dean in the 1930s.<sup>32</sup> Although the increase in prevalence has occurred primarily in very mild and moderate forms of dental fluorosis, there is evidence that the number of cases of moderate and severe fluorosis is increasing as well.<sup>2</sup>

Research on the prevalence and severity of enamel fluorosis can be divided readily into four distinct eras.<sup>33-35</sup> The focus of the first era was generated by the public health expediency of finding the cause of mottled enamel, and once found, of determining an acceptable level of fluoride in drinking water supplies. The second era began in the mid-1940s with community trials in which standards for water fluoride levels were adjusted. The third era, beginning in the mid-1970s, centered on the question of safe drinking water standards, primarily for water with above-optimal levels of fluoride.<sup>36</sup> The fourth era was delineated in a publication by Leverett,<sup>23</sup> who suggested that the fluoride burden might be reaching a critical level in the population, resulting in large benefits, but also in an increasing prevalence of fluorosis.

US residents have more sources of fluoride available now than 50 years ago. The success of water fluoridation in preventing and controlling dental caries led to the



development of fluoride-containing products, including toothpastes (i.e., dentifrice), mouthrinses, dietary supplements, and professionally applied or prescribed gels, foams, and varnishes.<sup>15</sup> In addition, processed beverages, which constitute an increasing proportion of the diets of many US residents,<sup>37,38</sup> and food can contain varying amounts of fluoride, especially if they are processed with fluoridated water.<sup>15</sup> The widespread use of fluoride in both fluoridated and non-fluoridated communities since the 1940s, and the “halo” effect (the diffusion effect of beverages and food processed in fluoridated areas but consumed in non-fluoridated areas<sup>15</sup>) have been some reasons given for the increase in the prevalence of fluorosis. Although technically a community that does not have fluoride in its water supply is considered non-fluoridated, in reality its population has access to other sources of fluoride such as the above-mentioned fluoride supplements and fluoride toothpastes, as well as fluoride in the food and beverages they consume.<sup>3</sup> For example, over 95 percent of the population in developed countries use fluoride toothpastes,<sup>39,40</sup> and foods and beverages may be processed in fluoridated communities but are shipped, sold, and consumed in non-fluoridated communities.

A review of the recent literature by Mascarenhas<sup>3</sup> in 2000 outlined the four major risk factors for the development of dental fluorosis quite well. The risk factors mentioned were fluoridated drinking water, fluoride supplements, infant formulas, and use of fluoride containing toothpastes. Dean<sup>41</sup> in his early studies recommending fluoridation of water estimated a 10-percent prevalence of mild or very mild fluorosis in the permanent teeth at water fluoride levels of 1.0 ppm. Reports of 10 years to 17 years after water fluoridation from the Newburgh-Kingston and Grand Rapids water fluoride studies showed that 7 percent to 16 percent of the children born and raised in the areas

exhibited dental fluorosis.<sup>42,43</sup> This degree of prevalence was recorded when drinking water was virtually the only source of fluoride. The current method of determining the optimal concentration of fluoride in community drinking water, which depends on the average maximum annual ambient air temperature, should be re-evaluated, because of the social and environmental changes that have occurred since the method was adopted in 1962.<sup>15</sup>

Fluoride supplements were also mentioned as a risk factor. Fluoride supplements are recommended for use in children in fluoride-deficient areas as a caries-preventive measure. Numerous studies<sup>44-46</sup> have shown that supplements are also prescribed to children in fluoridated areas, albeit inappropriately. In fluoridated areas, the risk of fluorosis from use of fluoride supplements is much higher, almost four times that in non-fluoridated areas. The supplement guidelines used in the studies reported by Mascarenhas,<sup>3</sup> were the older revised guidelines established in 1979. The increased risk of fluorosis demonstrated by the results of these studies caused a further revision of the fluoride supplement dosage in 1994, which was supported by the American Dental Association (ADA), the American Academy of Pediatric Dentistry (AAPD) and the American Academy of Pediatrics.<sup>47</sup> No studies have yet reported the risk of fluorosis for children born after the 1994 supplement guidelines who used supplements.<sup>47</sup> It is anticipated, however, that the risk of dental fluorosis will have been reduced.

In the Mascarenhas<sup>3</sup> article, infant formulas were also mentioned as a risk factor. Infant formulas in North America contained variable and often high concentrations of fluoride<sup>47,48</sup> until 1979 when the manufacturers voluntarily reduced and controlled the concentration of fluoride in their products. Studies of risk factors for fluorosis involving



children who were born before 1979 have shown infant formula to be a risk factor for fluorosis in fluoridated and non-fluoridated areas with statistically significant risk as high as seven times in the fluoridated areas.<sup>49-51</sup> However, concern continued that even after the reduction of fluoride content, infant formula was still a potential risk factor, particularly in fluoridated communities. Pendrys<sup>4,52</sup> and colleagues designed a series of studies to evaluate the risk of fluorosis in children born after 1979. These studies showed that in nonfluoridated communities, infant formula use was no longer a risk factor for dental fluorosis,<sup>52</sup> but in the fluoridated areas infant formula was still a significant risk factor.

The fourth major risk factor mentioned in the Mascarenhas<sup>3</sup> article was the use of fluoride toothpastes. The fluoride toothpaste variables that have been associated with fluorosis are: beginning toothbrushing at a relatively early age<sup>39,49,53,54</sup>; amount of toothpaste used measured as either toothbrushing frequency,<sup>55,56</sup> amount swallowed,<sup>54,57,58</sup> or the amount of toothpaste used at each brushing.<sup>59</sup> Although the association between toothpaste use and fluorosis was not always statistically significant,<sup>54</sup><sup>59,60</sup> the studies evaluated in this article taken together are compelling evidence that use of fluoride toothpastes before the age of six years is a risk indicator for dental fluorosis.<sup>3</sup> Fluoride toothpastes contribute to the risk for enamel fluorosis, because the swallowing reflex of children aged <6 years is not always well-controlled, particularly among children aged <3 years. Children are also known to swallow toothpaste deliberately when they like the taste.<sup>15</sup>

In a study by Pendrys,<sup>31</sup> the risk factors in both non-fluoridated and optimally fluoridated populations were summarized. Enamel fluorosis in the non-fluoridated study

sample was attributed to fluoride supplementation under the pre-1994 protocol and early toothbrushing behaviors. Enamel fluorosis in the optimally fluoridated study sample was attributed to early toothbrushing behaviors, inappropriate fluoride supplementation, and the use of infant formula in the form of a powdered concentrate.

It is important at this point to have an understanding of the definition, the established clinical manifestations, and the esthetic implications of dental fluorosis. “Dental fluorosis,” a specific disturbance in tooth formation and an esthetic condition, is defined as a chronic, fluoride-induced condition, in which enamel development is disrupted and the enamel hypomineralized.<sup>61</sup> Simply put, dental fluorosis is a condition in which an excessive amount of fluoride is incorporated in the developing tooth enamel.<sup>3</sup>

Specific diagnostic criteria, based upon established clinical signs, can be utilized to differentiate fluorotic from non-fluorotic enamel opacities.<sup>41,62-64</sup> In milder forms of enamel fluorosis, signs can include narrow white lines following the perikymata, cuspal snowcapping, and a snowflaking appearance that lacks a clear border with unaffected enamel. These markings are not readily apparent to the affected person or casual observer.<sup>15</sup> As the dental fluorosis severity increases, confluent areas of opacity become more evident with more pronounced mottling, staining, and pitting, with marked anatomic defects visible in the most severe cases. These increasing levels of clinically observed fluorosis severity are closely associated with underlying histologic changes in hypomineralization.<sup>41</sup>

While dental fluorosis does not have life-threatening implications, the esthetic ramifications are important, especially in our current esthetic-driven society. In a study by McKnight et al.,<sup>65</sup> it was shown that respondents generally found the images of



fluorotic teeth to be less esthetic. Clark et al.<sup>66</sup> analyzed responses from schoolchildren and their parents relative to concerns about the color of the child's teeth and related these subjective assessments to the different classifications of the Tooth Surface Index of Fluorosis (TSIF) (see Table III for the TSIF). Results suggested that for the majority of children with scores of 1, there were no esthetic concerns. Children and parents expressed more concern with fluorosis when TSIF scores were in the range of 2 to 6. These studies seem to indicate that the general public is able to recognize dental fluorosis and has esthetic concerns for what would be considered moderate to moderately severe fluorosis.

The development of fluorosis primarily affects mineralized tissues. It is important to note that approximately 99 percent of the fluoride contained in the body is associated with calcified tissues.<sup>67</sup> Fluorosis can affect the form, function, and esthetics of not only the teeth, but also the skeleton. The rate of skeletal uptake is affected by several factors including the level of fluoride intake and the hormonal status of the individual.<sup>68</sup>

Dental fluorosis has limited, if any serious medical consequences, whereas skeletal fluorosis can lead to severe crippling deformities. Bone, joint, and muscle pain, and progressive ankylosis of various joints characterize endemic skeletal fluorosis. Osteosclerosis and interosseous membrane calcification have long been regarded as hallmarks of this disease. Additional radiological findings include coarse trabecular pattern, and axial osteosclerosis with distal and diffuse osteopenia. Wang et al.<sup>69</sup> performed a radiographic survey of 127 patients with clinically proven endemic fluorosis and determined that skeletal fluorosis can have a wide variety of radiographic appearances, including calcification and ossification of the attachments of soft tissue

structures to bone, osteosclerosis, osteopenia, growth lines, and metaphyseal osteomalacic zones. Christie<sup>70</sup> performed a radiologic survey of 251 (<16 years old) Tanzanian children and found a wide variety of defects including knock-knees, bowlegs, saber shins, and combinations of osteomalacia and osteoporosis. Radiographic changes suggestive of rickets, hyperparathyroidism, and thalassemia can also be observed in skeletal fluorosis.

According to Kleerekoper,<sup>71</sup> fluoride appears to have two quite distinct effects on skeletal metabolism. Studies in humans and several animal models have shown that NaF stimulates bone formation. There is an increase in osteoid surface and thickness and an increase in matrix apposition rate.<sup>72,73</sup> The second mechanism whereby fluoride affects the skeleton lies in the affinity of the ion for the apatite crystal, replacing the hydroxyl group to form fluorapatite. The crystal lattice of fluorapatite is quite different from that of hydroxyapatite, and the changes render the crystal more stable.<sup>74,75</sup> This enhanced stability makes the skeletal structures more resistant to osteoblastic resorption,<sup>76</sup> further altering the normal remodeling cycle. Fluoride has profound effects on the skeleton, seemingly both beneficial and detrimental.<sup>71</sup>

At this point, a discussion on odontogenesis and the basics of enamel formation, amelogenesis, is important. It is also essential to understand normal enamel formation as well as how excessive amounts of fluoride can negatively impact the way enamel is formed. The following information on tooth development comes exclusively from two sources.<sup>77,78</sup>

The earliest signs of tooth development in the human embryo are in the anterior mandibular region when the embryo is five to six weeks old. Soon after this, tooth



development appears in the anterior maxillary region and progresses posteriorly in both jaws. The tooth is formed from ectoderm (one of the three germ layers) and ectomesenchyme (embryonic connective tissue). The ectomesenchyme is derived from the neural crest cells. It is important to note that development of teeth begins long before birth and continues long after birth. This prolonged development period means that the developmental process (cell differentiation, matrix production, mineralization) is susceptible to many different environmental stimuli (diseases, diet, drugs).

Four different stages of tooth formation are recognized: (1) lamina, (2) bud, (3) cap, and (4) bell. Development begins with the formation of primary dental lamina (stage one), during which a narrow band of thickened oral epithelium (ectoderm) extends along what will become the occlusal borders of the mandible and maxilla on a line where teeth will later appear. The enamel organ of each tooth forms from the dental lamina as a growth of oral epithelium into the underlying connective tissue. Each enamel organ is the beginning of the tooth germ of a tooth. The enamel organs are first knobs of epithelial cells and are known as buds (stage two). As the enamel organ enlarges, it acquires the shape of a cap (stage three). The cap undergoes further changes. The connective tissue inside the cap becomes the dental papilla, and the connective tissue beneath the dental papilla becomes fibrous and encircles the papilla and part of the enamel organ forming what is called the dental sac. In summary, a tooth germ is composed of three parts: (1) the enamel organ (composed of epithelial cells that arise from the ectoderm germ layer), (2) the dental papilla, and (3) the dental sac. The latter two parts form connective tissues that arise from mesenchyme.

As the tooth germ grows, the cap-shaped enamel organ changes form and becomes somewhat bell-shaped (stage four), and four layers are distinguishable. The four layers are:

- (1) The outer enamel epithelium: the outside layer of the enamel organ composed of low cuboidal cells.
- (2) The stellate reticulum: a layer immediately inside the outer enamel epithelium composed of a very loose network of epithelial cells.
- (3) The stratum intermedium: a layer of closely packed, flat epithelial cells inside the stellate reticulum.
- (4) The inner enamel epithelium: lines the inside of the enamel organ in a single layer of cuboidal cells. This layer of cells is separated from the dental papilla by a basement membrane.

The growing tooth germ changes form rapidly at first, but by the time the four layers of the enamel organ are well-defined, the shape of the basement membrane has become fixed. When the final shape of the basement membrane is established, it marks the line which will become the DEJ (dentino-enamel junction) of the finished tooth.

#### ODONTOGENESIS, ENAMEL FORMATION, AND THEORIES OF HOW ENAMEL FORMATION IS AFFECTED

Further cell differentiation in the tooth germ occurs along either side of the basement membrane. First, cuboidal cells that make up the inner enamel epithelium elongate into columnar cells called ameloblasts. Formation of ameloblasts is followed by a change in the peripheral cells of the dental papilla that also take a columnar form and become odontoblasts. The ameloblast and odontoblast layers are separated from each



other by the basement membrane. When ameloblasts and odontoblasts have differentiated along either side of the basement membrane in the tooth germ, formation of the hard tooth tissues begin. The earliest formation of the hard tooth tissues in the human fetus occurs during the fifth month in the primary incisors. Dentin formation starts just a little before enamel formation begins.

Amelogenesis is the name given to the origin and formation of enamel. Tooth enamel is a product of the enamel organ. The ameloblasts produce an organic enamel matrix in which mineral salts later crystallize out of solution, making enamel a hard tissue.

Amelogenesis consists of approximately three stages: (1) a secretory stage, (2) a post-secretory transitional stage, and (3) a maturation stage. Each of these stages is characterized by certain histological events. In each stage, the ameloblasts play a specific role as well. An overview of amelogenesis follows.

#### SECRETORY/SECRETION STAGE OF AMELOGENESIS

Secretory ameloblasts, like the odontoblasts, are polarized cells with a secretory or apical end and a non-secretory or basal end. They migrate in an outward direction away from the DEJ and secrete enamel. The initial or first formed enamel is aprismatic. During differentiation, ameloblasts acquire, among other things, a Tomes' process, a specialized apical process. Acquisition of a Tomes' process signals the beginning of the secretory stage of amelogenesis. As the ameloblast develops and acquires this Tomes' process, enamel rods (prisms) are formed.

## POST-SECRETORY TRANSITIONAL STAGE OF AMELOGENESIS

The stage of post-secretory transition occurs toward the end of enamel secretion and is marked by two developmental events. (1) A change in the morphology of the ameloblast takes place: ameloblasts lose their Tomes' process, and they become shorter. (2) Programmed cell death: many, up to 25 percent, of the ameloblasts die. A basal lamina and associated hemidesmosomes, which provide attachment to the enamel surface, are formed between the enamel and ameloblasts. The cells of the stellate reticulum become compact as well.

## MATURATION STAGE OF AMELOGENESIS

During the process of maturation, enamel becomes fully mineralized. The organic and water content of enamel becomes reduced, and the inorganic component (principally hydroxyapatite) increases. The process of maturation is really an ongoing process that begins early in the secretion stage. The enamel matrix becomes mineralized as soon as it is formed, and it continues to mature. This distinguishes it from dentin and bone, in that there is no "pre-enamel." The maturation process is continuous, but acquisition of full mineralization with loss of almost all the water and protein occurs during the stage of maturation. This stage is first recognized by the formation of a ruffled apical border in ameloblasts.

In the stage of enamel maturation, modulation (an activity of the ameloblasts) is an important concept to understand. It is a reversible change in cell activity and morphology.



There are two types of ameloblasts that participate in modulation.

(1) RE (ruffle-ended): possess ruffled distal border. These ameloblasts predominate during the maturation stage.

(2) SE (smooth-ended).

In the rodent incisor, where a rapidly growing and continuously erupting tooth often is used as a model for studying amelogenesis, it has been found that approximately 60 percent of the ameloblasts are RE, approximately 20 percent are SE, and approximately 20 percent are in various stages of transition between the two types.

RE ameloblasts bear a superficial resemblance to resorptive cells (e.g. intestinal villi epithelium). These RE cells may be responsible for the uptake of peptides and amino acids from the enamel matrix. The ruffle border also varies in appearance during maturation. It initially appears loaded with dark/dense staining material during early maturation and then becomes more vacuolated and dilated as the enamel matures.

The net result of the activity of maturation ameloblasts is a gain in the mineral content (mostly calcium and phosphate) of the enamel and a loss of protein and water. Spaces between enamel crystals diminish in size with the addition of more mineral to the matrix. The crystals of young enamel are long and slender. During maturation, the crystals get thicker and wider.

In summary, enamel mineralization follows the pattern of matrix formation from the DEJ peripherally. The extent of mineralization is indicated by the dark-to-light shaded zones proceeding from the DEJ peripherally. The very dark zones are the most highly mineralized, and the white areas, the least mineralized. The final stage of



mineralization of the enamel rod may be in its periphery and at a time just prior to eruption of the crown into the oral cavity.

## PROTEINS OF THE ENAMEL MATRIX

The enamel matrix is a complex mixture of proteins. Tissue-specific proteins (produced by ameloblasts) and cellular activities of ameloblasts during maturation are central to the development of this unique mineralized tissue. The proteins of the enamel matrix are classified as belonging to one of two major groups, (1) amelogenins, or (2) non-amelogenins.

Amelogenins are the predominant enamel matrix proteins. They comprise approximately 80 percent of the young enamel matrix. They are generally hydrophobic proteins with a hydrophilic sequence at their carboxy terminal (anionic) end. They contain high levels of the amino acids proline, glutamine, histidine, and leucine. Amelogenins have a tendency to aggregate in solution and to form supramolecular structures 20 nm in diameter called enamel nanospheres.

The crystals of mature enamel are by far the largest crystals found in mineralizing tissues of the body. Their growth in length, thickness, and width is controlled by their interaction with amelogenins during development. In developing enamel, amelogenin nanospheres electrostatically adhere to the developing enamel crystals. The initially thin hexagonal shape of the crystals is maintained by adherent amelogenin nanospheres. Crystal growth occurs along the c-axis (longitudinal axis) of the crystal by preferential deposition of mineral at the end of the crystal. Growth of enamel crystals, in width and thickness, is prevented or controlled by the presence of amelogenins and perhaps



enamelins on these surfaces. Besides directing growth, amelogenins have been proposed to serve as “20 nm spacers” to prevent premature fusion of crystals.

The role of non-amelogenins in crystal growth is less well understood. The major matrix proteins in this group are:

- (1) Tuftelin: Appears to be restricted to an area near the DEJ (in enamel tufts). Appears to be a product of both young ameloblasts and pre-odontoblasts. Due to its restricted location, it has been proposed to play a role in induction, the initiation of mineralization, and as a junctional matrix linking enamel and dentin.
- (2) Sheathlin: When it is initially secreted, it is found throughout the rod and interrod enamel. It is preferentially located in the rod sheaths or arcades in deeper enamel layers. In developing enamel, the rod sheath area is poorly mineralized.
- (3) Enamelin: It is an acidic, phosphorylated, and glycosylated protein. It is the largest enamel-matrix protein and is preferentially restricted to the enamel-rod area. Its phosphorylated nature and initial accumulation near the growing ends of crystals suggests that enamelin may play a role in crystal growth or nucleation. Similar to amelogenin, enamelin has also been proposed as a factor in limiting crystal growth.

## ROLE OF PROTEOLYTIC ENZYMES IN ENAMEL DEVELOPMENT

In addition to matrix proteins, hydrolytic enzymes have been found in the enamel matrix.

- (1) Enamelysin: It is a matrix metallo-proteinase. It is secreted with matrix proteins during the secretory stage of amelogenesis. It is probably responsible for



the limited proteolysis that occurs at this time. During post-secretory transition, production of enamelysin becomes reduced.

(2) Serine Proteases: The production of more aggressive serine proteases begins and continues throughout the maturation stage of amelogenesis. Serine proteases remove amelogenins from the inter-crystal areas, allowing growth in crystal width and thickness.

## EFFECTS OF FLUORIDE ON DEVELOPING DENTAL TISSUES

The effects of fluoride on dental tissue are fairly clear. It is well established that a linear relation between the dose of fluoride and the severity of enamel fluorosis in humans exists. Fluoride interacts with mineralized tissues and at elevated concentrations disturbs the mineralization process. As exposure to fluoride is increased during tooth formation, the subsurface enamel all along the tooth becomes increasingly porous (hypomineralized) extending toward the inner enamel. Hypomineralization of the dentin results in an enhancement of the incremental lines. Following tooth eruption, the more severe forms of fluorosis are subject to extensive mechanical breakdown of the surface.<sup>61,79,80</sup>

In a recent review of animal studies performed by Richards,<sup>81</sup> two distinctly different types of enamel lesions were characterized in animals. These lesions could be produced by long-term exposure to fluoride. The size, duration, and timing of the fluoride dose in relation to the stage of enamel formation determine which types of lesions occur. Low doses produce sub-surface hypomineralization of the enamel without alterations in the basic structure of the tissue. Higher doses that commence prior to the



enamel maturation stage produce true hypoplastic lesions in addition to hypomineralization.

In addition to systemic exposure to increased fluoride, the development of fluorosis is coupled to pre-eruptive tooth maturation.<sup>82</sup> The risk of developing enamel fluorosis appears to be greatest when exposure occurs during both secretory and maturation stages of enamel formation.<sup>83-86</sup> The subsurface porosity is thought to be caused by a delay in the hydrolysis and removal of enamel proteins, particularly amelogenins as the enamel matures.<sup>87</sup> Recently, Kirkham et al.<sup>88</sup> used atomic force microscopy to image and quantify surface features of enamel crystals isolated from specific developmental stages of fluorotic and control rat incisors. The effect of fluoride on enamel crystal surfaces was seen in the nature and distribution of growth sites and in mineral-matrix interactions. These changes would be expected to affect crystal growth during maturation, resulting in the characteristic porous appearance of fluorotic lesions in mature teeth. To date, however, a clear understanding of the pathogenesis of enamel fluorosis is not known.

In a systematic review of the dental literature (1966-1998) by Bardsen,<sup>89</sup> risk periods associated with dental fluorosis in the maxillary permanent central incisors were investigated. Of the 10 studies included in the review, they were categorized into two broad groups:

Group One: Subjects whose exposure to fluoride started at different ages during enamel formation.



Group Two: Subjects who had been exposed from birth and then experienced an abrupt reduction in daily fluoride exposure at different ages during the amelogenesis period.

The conclusions of the study included: (1) There was no single period of enamel formation that could be singled out as being the most critical for the development of dental fluorosis, and (2) The duration of fluoride exposure during the amelogenesis is most critical, rather than specific risk periods.

In another study by Bardsen in 1998,<sup>90</sup> 40 children who had been life-long consumers of moderate- to high-fluoride drinking water were examined as well as their older siblings who were born six months to six years before the fluoride containing drinking water was introduced to the household. As compared with their older siblings, the prevalence of dental fluorosis was significantly higher in the children who had consumed moderate- to high-fluoride water throughout their lives. The findings of the study indicate that early mineralizing teeth (central incisors and first molars) are highly susceptible to dental fluorosis if exposed to fluoride from the first year, and to a lesser extent, the second year of life.

Epidemiologic indices for measuring the clinical manifestations of dental fluorosis have been created.<sup>91</sup> Three indices (1) Dean's Index,<sup>92 62</sup> (2) Thylstrup and Fejerskov Index (TFI),<sup>41 93</sup> and (3) Tooth Surface Index of Fluorosis (TSIF)<sup>64</sup> are commonly used today. The original criteria for the Dean's Index were developed in 1934 and later modified in 1942. The index provides a scale from 0 (normal) to 4.0 (severe). Dean's Index can be seen in Table I. The original criteria for the TFI index were developed in 1978 and later modified in 1988. The TFI provides a scale from 0 to 9. The



TFI has been appealing to epidemiologists and clinicians because its classification scale correlates well to histological changes that take place in dental fluorosis,<sup>41</sup> and also to the fluoride concentrations found in enamel.<sup>81</sup> The TFI can be seen in Table II. The TSIF was developed in the early 1980s, and it provides a scale from 0 (normal enamel) to 7 (severely affected enamel). The TSIF can be seen in Table III.

Each of the three indices listed above have proven useful in epidemiological and clinical trials. Kumar et al.<sup>94</sup> utilized Dean's fluorosis index in a study involving fluorosis examinations on 202 children in Newburgh and Kingston, NY. In conclusion, examiners showed good to excellent agreement beyond chance in the use of the index. In another study by Pereira and Moreira,<sup>95</sup> the objective was to compare the Dean's, TF, and TSIF dental fluorosis indices in relation to the prevalence of surfaces, teeth, and locality, and to verify the statistical correlation among them. They concluded that there were no difficulties in using the three indices in the field trials; thus the use of any one may be recommended in regions with similar fluoride concentrations to those of this study.

In addition to the Dean's, the TFI and the TSIF, two other indices have also been developed. The fourth index is the Fluorosis Risk Index (FRI) and has been developed by Pendrys.<sup>96</sup> In any study of the association between fluoride exposure and fluorosis, it is important to relate time of exposure closely to the period when enamel is at risk. The FRI has been designed to address this linkage problem and to respond to the current pattern of fluoride exposure in US children, which can be highly variable during the period when teeth are at risk of fluorosis. The unique feature of the FRI is that each tooth is divided into zones that correspond to the age at which they begin development and can



be related to narrow age-bands of fluoride exposure, such as a 12-month time period. The Fluorosis Risk Index can be seen in Table IV.

The fifth index is the DDE, the Developmental Defects of Enamel Index. The DDE was initially created in 1982.<sup>97</sup> In the DDE, the type (opacity, hypoplasia, discoloration), number (single and multiple), demarcation (demarcated and diffuse), and location of defects on the buccal and lingual surfaces of teeth could be recorded. However, the large amount of data generated had caused difficulties in presenting results in a meaningful fashion, in interpreting results, and in making comparisons between studies.<sup>98</sup> A modified DDE was presented by Clarkson and O'Mullane in 1989.<sup>99</sup> The results of that study reported that the refined DDE Index could be used to measure effectively the more important types of enamel opacities, including their color, their demarcation (whether demarcated or diffuse in nature), and hypoplastic defects. The modified DDE Index can be seen in Table V. A study by Holt et al.<sup>100</sup> utilized effectively the TF index for fluorosis and the modified DDE index to score enamel opacities in children between two to five years of age.

Dental fluorosis results from an environmental challenge of greater than optimal amounts of fluoride taken systemically. Fluorosis can be described as a complex process where variation in severity exists in the population even though exposure to fluoride can be quite similar. In a human study performed by Dr. Karen Yoder<sup>101</sup> in collaboration with Dr. Lameck Mabelya, children were identified who had very little evidence of enamel disturbances despite the fact that they had lived in areas of endemic fluorosis (prevalence of 100 percent) for their entire lives. There was no ready explanation for the lack of dental fluorosis in these children.



In this Tanzanian population, Dr. Lameck Mabelya was able to determine that the use of a high fluoride-containing trona tenderizer (magadi) was contributing to dental fluorosis. Dr. Mabelya's<sup>102</sup> findings were outlined in his 1997 publication and are summarized here. He selected 18 villages in four geographical areas (districts) with water supplies containing 0.2 to 0.8 mg/L of fluoride. All the schoolchildren aged 12 to 17 years (n = 1566) who had been born and raised in these villages were examined for dental fluorosis according to the Thylstrup-Fejerskov Index (TFI). Dietary history was recorded.

Nine coastal villages were selected as well as nine villages located inland. The prevalence of dental fluorosis in the nine coastal villages where tea and seafish were regularly consumed ranged from 7 percent to 46 percent. Severe (pitting) dental fluorosis was rarely seen. The low fluorosis levels observed in non-magadi consuming communities in coastal villages indicate that a fluoride content of up to 0.8 mg/L in drinking water is acceptable under the prevailing conditions of temperature and diet.

In contrast, the prevalence of dental fluorosis in the inland villages at 1500-m altitude, where fluoride-containing magadi was consumed, ranged from 53 percent to 100 percent, and severe (pitting) fluorosis was highly prevalent ranging from 18 percent to 97 percent. The village with the highest fluoride content in the magadi samples collected showed the highest level of fluorosis. Data on dental fluorosis from the magadi-consuming communities provide strong evidence that consumption of magadi was the major determinant of the observed high prevalence and severity of fluorosis in inland villages at 1500-m altitude.



In regard to the above mentioned Tanzanian children who had lived in areas of endemic fluorosis and showed little evidence of fluorosis, the question to be asked is, "Are there genetic determinants that contribute to susceptibility/resistance to fluorosis?" Animal models, specifically several inbred mouse strains, have been chosen to address this issue. Mice have not been extensively utilized in studies on fluorosis. Rats, on the other hand, have been used quite extensively.<sup>84,85,103-108</sup> One example of the utilization of rats can be found in a 1984 journal article, *Enamel Fluorosis Related to Plasma Fluoride Levels in the Rat*.<sup>103</sup> One of the findings of this study was that "the rat incisor is a better model for human enamel fluorosis than heretofore believed."

Having said that, a question that begs asking is, "How do the rat and mouse compare with one another?" In an article by Coady et al.,<sup>109</sup> *Histology of the Mouse Incisor*, a direct comparison was made between the incisor of the rat and the incisor of the mouse. It was concluded that a significant structural similarity exists between the maxillary incisor of the mouse and that of the rat. Also, turnover time of the mouse incisor is about the same. The only notable difference is, of course, in size. It is safe to say that mice as well as rats can be utilized in studies of fluorosis.

Animal models must be used, because, *in vivo* studies in humans are not possible due to the ethical considerations and implications. "Opportunities to study human material, such as the accidental ingestion of a fluoride-containing material by a child and the subsequent recovery of the teeth, must be very rare."<sup>104</sup> In this 1979 publication by Fejerskov et al., one of the researchers (J.Y.) had access to specimens prepared by the late Dr. Isaac Schour. The material was from planned experiments in which children with inevitable fatal inoperable hydrocephalus were injected intravenously with sodium



fluoride at a dose of about 5mg/kg body weight. The injections of fluoride were repeated at about weekly intervals for approximately four months. When these infants subsequently died because of the hydrocephalus, their teeth were recovered.

In conclusion, it was found that acute and chronic doses of fluoride giving rise to lesions in rat incisors and in human teeth are essentially identical. Studies like this one involving humans could not be performed today. However, the information it gives us is that humans and rats respond similarly to fluoride in the lesions that develop; because rats and mice are nearly identical in their incisor histology, mice, too, should produce fluorotic lesions similar to those in humans. Therefore, mice are useful in studies of the effect of fluoride on tooth development.

There are a number of significant reasons for choosing the mouse as an animal model. Inbred mouse strains are easily accessible and available as normal laboratory strains. They have a very well-defined genetic makeup. They are very different and demonstrate remarkably different phenotypes. Many inbred strains are bred for specific phenotypes.<sup>110</sup>

An inbred mouse strain is described in this way, "a strain shall be regarded as inbred when it has been mated brother x sister (hereafter called bxs) for twenty or more consecutive generations (F20), and can be traced to a single ancestral breeding pair in the 20<sup>th</sup> or a subsequent generation."<sup>110</sup> At 20 generations, on average at least 98.6 percent of the loci in each mouse are homozygous. Many strains have been bred for more than 150 generations and are essentially homozygous at all loci. Each inbred strain is also isogenic (genetically identical), because all individuals trace back to a common ancestor



in the 20<sup>th</sup> or a subsequent generation.<sup>110</sup> This feature shared with F1 hybrids makes it possible to build up a genetic profile of the strain by typing an individual.<sup>111</sup>

Twelve inbred mouse strains were utilized in this research project and were selected based upon their degree of unrelatedness from each other. Each inbred mouse strain represented a genetically homogenous group that often differs quite remarkably from mice in another inbred strain. See Figure 1, a genealogy of 24 inbred strains of mice taken from Atchley, 1991.<sup>112</sup>

Inbred strains have been used for immunologic and genetic studies because of the isogenicity within a strain and the genetic heterogeneity between inbred strains.<sup>110,113,114</sup> “Inbred strains of rodents represent fixed, unique genotypes that can be repeatedly accessed as homogeneous populations, with defined allelic composition and predictable phenotypes. As such, they represent a remarkable experimental resource; the consequences of genetic or environmental manipulations are measurable against a constant genetic background, providing a nearly ideal setting for the analysis of complex physiological processes, including virtually all the common human diseases.”<sup>115</sup> This is an incredible statement by Paigen indicating the immense importance of inbred mouse strains in genetics research.

Mice have a short life span and continuously erupting incisors. Coady et al.<sup>109</sup> established that a mouse maxillary incisor has an average weekly rate of eruption of 1 mm. Meyer et al.<sup>116</sup> concluded that a mouse mandibular incisor has an average weekly rate of eruption of 1.7 mm. The rate of the eruption both of the upper and lower incisors of the mouse is about 1 mm per week less than that of the rat. The average length of the mouse maxillary incisor is 7.9 mm. At an average rate of eruption of 1 mm per week, it



is estimated that the complete replacement of the incisor takes place in 7.2 weeks or 50.4 days.<sup>109</sup> Therefore, environmental changes can be measured longitudinally. The continuous growth and eruption of the mouse incisor, as well as a high turnover rate, provides a very useful model for studies of amelogenesis.

Coady<sup>109</sup> also found that, grossly, the enamel surface of the mouse incisor showed yellow-brown pigmentation. In addition, Amar in 1989<sup>117</sup> found that mouse incisor tooth organs express the genes responsible for enamel extracellular matrix formation exclusively on the labial surface of the organ. Hence, the mouse incisor only exhibits enamel on the labial/facial surface of the tooth.

Moinichen et al.<sup>118</sup> listed some important morphological characteristics of mouse incisor enamel. The general design of the enamel structure resembled rat incisor enamel with a uniserial lamellar pattern of prisms in the inner enamel and incisally directed parallel prisms in the outer enamel. The centrolabial thickness of the enamel was about 60  $\mu\text{m}$  in the maxillary and about 95  $\mu\text{m}$  in the mandibular incisors. Ameloblasts in mouse mandibular incisors produce enamel at a rate of about 6  $\mu\text{m}$  per day, about half the corresponding rate in the rat. In spite of this, the mouse mandibular incisor has a relatively thick layer of enamel, because the ameloblasts spend a relatively long time in the zone of enamel secretion due to a fairly slow eruption rate.<sup>118</sup>

Despite their small size, mouse incisors are amenable for clinical assessment of fluorosis and with some modification, indices like Dean's and TFI can be applied. In a study by Kerley,<sup>7</sup> mice were fed fish protein concentrate (FPC). The FPC contained large amounts of fluoride and the effect on the dentitions of these mice was studied. "The first appearance of pigment abnormalities indicative of fluorosis in the incisors of the FPC-fed



mice was observed at eight weeks. The lateral and medial labial borders were bleached of pigment, with alternating light and dark orange bands confined to the middle labial enamel surface.” This article confirms that mice are amenable to studies of fluorosis. It also points out changes that are seen in fluorotic mouse enamel.

Quantitative Light-Induced Fluorescence (QLF), a non-destructive method used to detect and quantify the severity of carious lesions in enamel, can be investigated as a tool to quantitate enamel changes accompanying the onset of fluorosis in mice as well. The QLF has been used in various studies on dental caries, for measurements of demineralization and remineralization of enamel *in vitro*,<sup>119-121</sup> *in vivo*,<sup>122</sup> and has proved useful for detection of caries lesions in rat molars.<sup>123</sup>

The methods of QLF were discussed in detail in an article by de Josselin de Jong.<sup>119</sup> In Quantitative Laser-Induced Fluorescence (QLF), a tooth was illuminated with a broad beam of blue-green light (wavelength 488 nm) from an argon ion laser. The fluorescence in the enamel occurring in the yellow region was observed through a yellow high-pass filter to exclude the tooth-scattered blue laser light. A CCD (charged coupled device) camera was equipped with the high-pass filter and collected the fluorescent image of the teeth. A PC program was used for display, storage, and subsequent analysis of images. The QLF system has been modified<sup>124</sup> and currently uses an arc lamp, filtered to a small band ( $370 \pm 80$  nm). This system is integrated into a hand-held intraoral camera, by which an image of the area of analysis is saved. This modified system is called Quantitative Light-Induced Fluorescence (also abbreviated QLF). System integration and software are similar to the laser-based version of QLF.



Cariious lesions show up on the QLF images as a dark spots against a background of the brighter surrounding sound enamel, indicating a loss of fluorescence. The source of the fluorescent signal in the enamel has been identified in the organic portion of the enamel,<sup>125</sup> but much of the signal is based on having an underlying dentin lesion.<sup>126</sup> Dental fluorosis has been observed on images during caries examinations with the QLF technique, and present as diffuse dark areas.<sup>127</sup> Due to the nature of fluorosis in human teeth, as increased porosity occurs in the outer layers of the enamel, there is a good possibility that QLF may prove useful for the purpose of monitoring the onset and severity of dental fluorosis. Angmar-Mansson<sup>128</sup> proposed that the laser fluorescence method might be developed into a method applicable for the assessment of the severity of enamel fluorosis.



## MATERIALS AND METHODS

This study involved 12 separate inbred strains of mice. Each strain was chosen based upon maximum potential genetic unrelatedness.<sup>110</sup> Each strain involved three groups of animals and the study was conducted for 60 days with termination of treatment after 60 days. This study was approved by the Indiana University School of Dentistry Institutional Animal Care and Use Committee (study # DS0000627). The rationale for the choice of fluoride concentrations and delivery was based upon earlier work using rats.<sup>129-131</sup>

## MICE

Weanling (3-week-old) male mice representing the following inbred strains: 129P3/J, A/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BL/10J, CBA/J, DBA1/J, DBA/2J, FVB/NJ, SJL/J, and SWR/J were obtained commercially from The Jackson Laboratory (Bar Harbor, ME). The selection of these mouse strains was based upon their phenotypic and genetic differences (the degree of unrelatedness from each other) as well as their general availability as normal laboratory strains. The selection of juvenile mice allowed for the possibility to detect skeletal changes, if any, as the animals matured into adulthood.

Each of the three treatment groups consisted of 72 mice each (6 each from the 12 strains studied). Group 1 received distilled water only; Group 2 received distilled water supplemented with 25 ppm fluoride, and Group 3 received distilled water supplemented with 50 ppm fluoride. Mice were uniquely identified and housed 3 per box. The



examiners, ETE and MM were blinded to the treatment groups. All mice were monitored daily for any changes in health status.

## FLUORIDE TREATMENT

Three levels of fluoride treatment were delivered in the drinking water (0 ppm, 25 ppm, and 50 ppm fluoride). The deionized and prepared water was periodically analyzed by using an ion specific electrode (ISE) for fluoride concentration. The deionized water tested from multiple sites within the Bioresearch facility showed  $[F] = 0.018 \pm 0.01$  ppm. Each batch of prepared 25 ppm and 50 ppm water was analyzed and  $[F] = 24.487 \pm 0.270$  ppm and  $49.680 \pm 0.506$  ppm, respectively.

Species specific differences in the effect of fluoride on mineralized tissues have been observed for humans and rodents (primarily rats). Earlier studies suggest that in order to see an observable change in rodents, fluoride exposure should be about 10 times that needed to produce a similar effect in humans. The rationale for determining the range of fluoride concentrations in this project was an extrapolation of earlier work using rats.<sup>129-131</sup> It is correct that community water fluoridation results in  $[F]$  around 1 ppm to 2 ppm, which has been proven to be safe and effective. Therefore, mice would require 10 ppm to 20 ppm fluoride in the water to net similar effects as 1 ppm to 2 ppm has on humans. Our selection of the fluoride concentrations was partially driven by the desire to assure that fluorosis could be observed in the high concentration of 50 ppm given the relative differences between humans and rodents.

Pilot analyses found that mouse chow (Purina LabChow 5001) contains fluoride. Mice were fed *ad libitum* laboratory rodent diet #5001 (PMI® Nutrition International, Richmond, IN). Periodic fluoride determinations of different batches of mouse chow



used in the study were performed using a modified diffusion method.<sup>132</sup> The fluoride concentration ranged from 5 ug/gm to 8.037 ug/gm ( $7.229 \pm 0.796$  ug/gm). The preparation of fluoridated water and the care of mice used during this study was the responsibility of the Indiana University School of Dentistry (IUSD) Bioresearch facility. Determinations of fluoride content in the water, food, and mineralized tissues were through collaboration with Dr. Angeles Martinez-Mier in the Oral Health Research Institute (OHRI).

## ORAL EXAMINATIONS

Once a week each mouse was given a complete oral examination where incisor tooth color (the normal color of mouse incisors is deep yellow-orange, which is imparted by an iron containing pigment produced by the ameloblasts) was assessed along the entire labial surface of the enamel using a shade guide (ceramic custom coloring opaque powder shade guide, Vident, Brea, CA). The shade guide was analyzed using the CMYK (cyan, magenta, yellow, and black) color model in the Photoshop 5.5 software permitting the selective grouping of shades based upon yellow content. This reduced the observations to an ordinal scale (1-3) where 1 = predominantly shades of white or light tan and 3 = shades of yellow to yellow-orange. Opacity, length (in mm using a periodontal probe), wear pattern, and overall development of the upper and lower incisors were also independently judged. The determination of dental fluorosis was made clinically over the entire mandibular incisor tooth surface using a modified Thylstrup and Fejerskov index (TFI).<sup>41</sup> Grossly, the upper incisors and lower incisors of mice with dental fluorosis appeared white, chalky, and with thin striations and erosions of the enamel.



## EUTHANASIA OF MICE

At day 60, mice were taken from the Bioresearch Facility to DS270 where they were weighed, anesthetized (ketamine and xylazine, 70 mg/kg ketamine and 14 mg/kg xylazine), examined, and photographed. During anesthesia, the mice were euthanized by exsanguination and by major internal organ removal. During euthanasia, selected mineralized tissues were removed. Selected long bones were removed and frozen for fluoride analysis. The pairs of mandibular incisors were removed for QLF and for fluoride analysis (stored at -20°C).

Samples collected following anesthesia and euthanasia were assigned random numbers (from 1 to 216, the code was kept by ETE). Clinical material (images and radiographs), incisor QLF, bone ash weights, and hard tissue fluoride analyses were performed on samples in numerical ascending order. Only when all data had been acquired was the code broken to permit correlation between different strains and different treatment groups.

## CLINICAL IMAGES

Digital and 35 mm photography of the upper and lower incisors were performed on a random subset of mice during the course of the treatment period and on all mice at the conclusion of treatment (at termination). All animals were assigned a random number between 1 and 216. Each of the 216 mice was from one of 12 genetically distinct strains of mice. The coded images were subjected to clinical assessment for dental fluorosis.



Potential examiner bias was minimized by blinding the examiners to the identity of each of the mouse images.

Following a period of initial calibration using standard human dental fluorosis images and representative mouse images, the two examiners, MM and MF (Michael French, a dental student at IUSD), independently scored coded images using first the Modified Thylstrup and Fejerskov Index (Table II), and secondly the Dean's Index of fluorosis (Table I). The images were scored in ascending numerical order. Both the 35 mm slides and digital images were used, with from two to five images of each mouse per set.

A light box with an 8-10X monocular lupe was used for the 35 mm slides. The digital images were compiled onto a CD and were scored using a Compaq laptop computer with Adobe Photoshop 6.0 and a 14-inch monitor to view the digital images. The examiners were also calibrated randomly yet frequently throughout the project by discussing differences in scores to determine the correct classification of each.

After all of the images were scored, the code was broken and a series of correlations was performed between Observer 1 (MF), Observer 2 (MM), fluoride exposure, and fluorosis indices using the Pearson Correlation Coefficient (a measure of linear association between two variables).

## SKELETAL RADIOGRAPHY

Because the adverse effects of fluoride impact mineralized tissues, it was reasonable to investigate any possible changes to the skeleton. With this in mind, we chose to enroll mice at a young age (weanling) and assess the effects of fluoride on the skeleton well into adulthood (~13 weeks of age). Radiographs were an important



component in the evaluation of skeletal fluorosis. Therefore, we proposed to investigate any gross skeletal changes using standardized radiography. All mice were subjected to full skeletal surveys and skull radiographs from two views (dorsoventral and lateral cephalogram).

Full skeletal surveys were performed on euthanized mice using two pieces of size 4 Kodak Ultraspeed DF-50 occlusal film taped together. Each body was subjected to a voltage output of 20 kVp with an exposure time set for 10 seconds using a Hewlett-Packard Faxitron b Model 43855-A02 (Hewlett-Packard-McMinnville Division, McMinnville, OR) with a film to source distance of 61 cm, X-ray tube current 3 mA, with continuously variable voltage output 10-110 kVp. All skeletal radiographs were coded with the unique animal number and examined blindly using a 10X lupe over a standard light box for extra skeletal calcifications and other gross bony deformities.

#### FLUORIDE ACCUMULATION IN FEMURS AS AN INDICATOR OF EXPOSURE

The right femur was collected from all mice studied. Bone ash was prepared on the isolated femurs for total fluoride content. Each sample was placed in a labeled, pre-weighed silica crucible, covered and ashed in a muffle furnace (Barnstead/Thermolyne, Model No. PA 1740: Dubuque, Iowa) at 600 °C for four to five hours. Weight of the crucible plus the tissue ash was recorded. The samples were then placed individually in small, labeled plastic vials and the ash pulverized using a clean, glass rod. The pulverized, ashed samples were stored at -20 °C until analyzed. The powdered femurs were subjected to fluoride analysis using a modified diffusion method.<sup>132</sup>



## ANALYSES OF FLUORIDE

Deionized and fluoridated water used to treat mice were analyzed directly for fluoride concentration by Dr. Angeles Martinez-Mier, OHRI, using a combination of a fluoride-specific electrode (Orion #96-909-00) and an Accumet 950 pH/ion meter (Fisher Scientific). Analyses of mouse mineralized tissues and mouse chow were performed by Dr. Angeles Martinez-Mier, OHRI, using a modification<sup>132</sup> of the hexamethyl-disiloxane (HMDS: Sigma Chemical Co., St Louis, MO 63178), microdiffusion method of Taves.<sup>133</sup>

## QUANTITATIVE LIGHT INDUCED FLUORESCENCE (QLF) ANALYSIS

At harvest, one pair of mandibular incisors from each animal were dissected away from the hemi-mandibles, cleaned of gingiva, rinsed briefly in 10-percent buffered formalin, placed in a labeled microfuge tube, and placed in storage at -20 °C. For QLF analysis, the pair of incisors were wiped dry and placed labial side up and flat on a piece of black cardboard. A QLF camera handpiece was attached to a custom made stand at a fixed distance of 5 cm above a black cardboard plate. The mice teeth were laid flat on the cardboard and stabilized with wax if necessary. An image was taken of the teeth with the QLF handpiece connected to a control box (Inspektor Research Systems BV, Amsterdam, Netherlands) and analyzed using QLF software, version 2.00d. The software captured an image of the teeth using 10 frames to create the image, with the original image stored in a bitmap format.

The areas of the teeth that were affected by fluorosis on visual examination showed up as bright areas on the QLF images, indicating an increase in fluorescence.



The images were processed in an image processing software, by inverting the original images so that white lesions appeared dark. The inverted images were then analyzed using the algorithm in the QLF software, version 2.00d, to quantify the average increase in fluorescence and the size of the affected area of the teeth.

## RESULTS



## CLINICAL FLUOROSIS SEEN IN MICE PARALLELS FLUOROSIS IN HUMANS

The normal color of mouse incisors varies from strain to strain, but shades of yellow-orange are typical and are due to deposition of iron in the enamel.<sup>118,134,135</sup> The determination of dental fluorosis was made clinically over the entire incisor tooth surface using a modified TFI scale. When dental fluorosis developed, the upper incisors and lower incisors appeared white, chalky opaque, and thin. Striations and erosions of the enamel were evident and particularly prominent on the lower incisors (Figure 2, Panel A). As fluorosis continued to develop, the lower incisors became more opaque and resembled mild to moderate fluorosis in humans (Figure 2, Panel B).

Severe dental fluorosis was seen in the A/J strain, Figure 3. Dental fluorosis in the A/J strain developed early (within a couple of weeks) and was manifested at both the 25 ppm and 50 ppm [F] treatment groups. In contrast, the 129P3/J strain (Figure 4, Panels A, D) was the most resistant strain tested, showing minimal dental fluorosis only at the 50 ppm [F] level. The maxillary and mandibular incisors shown in the 0 ppm [F] panel illustrate normal tooth development at 13 weeks of age for both A/J and 129P3/J mice.

Weekly oral examinations of study mice showed that the onset of dental fluorosis was gradual in some strains (i.e. SWR/J, BALBc/J, C57BL/10J, and DBA/2J). Other strains (i.e. A/J, SJL/J, C57BL/6J, and C3H/HeJ) developed dental fluorosis more quickly. A few mouse strains like 129P3/J and CBA/J developed minimal dental



fluorosis. While all strains developed varying severities of dental fluorosis at 50 ppm [F], some strains were also responsive at the 25 ppm [F] level.

#### UPPER INCISOR COLOR AS AN INDEX OF FLUOROSIS

The natural color of the upper incisors was modified during the 60-day course of fluoride treatment, an effect that provided a semi-quantitative estimation of the onset and severity of fluorosis in each strain. Throughout the treatment period, tooth color changes were assessed by comparing upper (occasionally lower) incisor tooth color to a standard shade guide, a ceramic custom coloring opaque shade guide (Vident, Brea, CA), see Figure 5. Tooth color was assessed along the entire labial surface of the enamel.

To facilitate analysis of the longitudinal data, ETE and MM analyzed the shade guide using the CMYK color model in the Photoshop 5.5 software. The CMYK model is based on the light-absorbing quality of ink printed on paper. As white light strikes the color, part of the spectrum is absorbed and part is reflected back to the examiners' eyes. CMYK analysis of the shade guide resulted in the selective grouping of shades based upon yellow content, Table VI. This allowed us to reduce the observation to an ordinal scale (1-3) where 1 = predominantly shades of white or light tan, and where 3 = shades of yellow to yellow-orange.

Reducing the color scale to a range of 1-3, the examiners' observations highly correlated with each other. Inter-examiner (ETE and MM) variation for upper incisor color determination was measured using Pearson's correlation for each strain,  $r = 0.649$  to  $0.832$  (mean =  $0.740 \pm 0.06$ ) and was significant at  $p < 0.01$ , two-tailed. The variation of fluorosis development between strains and the dose response between fluoride exposure and diminished color development is shown in Figures 6a, 6b, and 7.



Monitoring these tooth color changes added dimension to the clinical characterization of dental fluorosis in these strains of mice.

The longitudinal study of upper incisor color permitted mice to be grouped into three phenotypic groups: resistant strains (129P3/J, FVB/NJ, CBA/J, and DBA/1J); intermediate strains (SWR/J, BALBc/J, C57BL/10J, and DBA/2J); and sensitive strains (A/J, SJL/J, C3H/HeJ, and C57BL/6J). Some strains exhibited a dose response (C3H/HeJ, SJL/J, and SWR/J), Figure 6a and (BALB/cJ, C57BL/6J, and C57BL/10J), Figure 6b, across both [F] and others responded best to 50 ppm only (e.g. CBA/J), Figure 6a. A few strains, including CBA/J (Figure 6a), FVB/NJ (Figure 6b), and DBA/2J (Figure 6b) appeared to normalize in color towards the end of the treatment period. Some strains tended to share similar patterns (e.g. C57BL/6J and C57BL/10J). Two strains, A/J and 129P3/J, constituted the extremes in sensitivity/susceptibility and resistance to fluorosis, Figure 7. A/J mice developed fluorosis early and responded to 25 and 50ppm [F]. These two strains would be suitable for QTL mapping.

## CLINICAL IMAGES

The correlation between fluoride exposure and the fluorosis scores was not high (Tables VII and VIII), but it was still significant. Using the observed scores from Observer 1 (MF) and Observer 2 (MM), both indices were highly correlated (Tables VII and VIII) in all aspects of the analyses. This showed that both indices were useful for classifying fluorosis in mice. In addition, it could be interpreted that neither index was superior to the other in classifying dental fluorosis. However, the upper end of the scale in both indices was rarely used for the mice. This is likely due to greater severity in human dental fluorosis, and these indices may be better suited for human classification.



Both examiners, MF and MM found it much easier to work with the digital images than the 35 mm slides. This is possibly due to the larger digital image size and the ability to zoom in on the image. This is reflected in the higher correlation between observers seen with the digital images over the 35 mm slides (Table IX). The higher correlation between the digital images and fluoride exposure versus the 35 mm and fluoride exposure also suggests that the digital images are potentially superior in this type of study. From Table IX it is also seen that inter-observer correlation was high. This can probably be attributed to the high frequency of calibration between observers.

While there is definitely a correlation between the amount of fluoride exposure and the fluorosis scores, these indices are not promising indicators of the fluoride burden during amelogenesis.

#### SKELETAL RADIOGRAPHY

All films were examined by ETE and MM, who were blinded to both the strain and treatment group. No gross skeletal lesions or evidence of bone dysplasia were noted. However, mild effects consisting of small ectopic calcifications around the pelvis and base of tail were present in three of the six SJL/J mice treated at  $[F] = 50$  ppm, Figure 8.

#### FLUORIDE ACCUMULATION IN FEMURS AND INCISORS AS INDICATORS OF EXPOSURE

Differences in dental fluorosis between mouse strains begged the question of whether total fluoride burden was different among the strains examined. Previous studies using rats suggested that monitoring water intake was not necessary.<sup>132</sup> During the pilot study, all mice appeared well-hydrated. The question remained regarding adequate



exposure and accumulation of fluoride in the mineralized tissues. We chose to assess body fluoride burden by determining the quantity of fluoride in femurs and mandibular incisors (Tables X and XI). The right femur was dissected from each mouse in the pilot study as well as the mandibular incisors.

Pearson's Correlation, two-tailed, was significant between fluoride concentration in water and fluoride concentration in the femur,  $r = 0.869$ ,  $p < 0.00001$ . A dose response also was noted between fluoride in the water and fluoride in the bone, Table XI. Overall, mice accumulated similar amounts of fluoride in their long bones regardless of whether or not they exhibited mild to severe dental fluorosis. Small but significant differences were appreciated at 25 ppm.

Because the erupted mandibular incisors have enamel covering only the labial surface, and because it averages 0.01 mm thick, it was not possible to separate the enamel from the dentin in our fluoride determinations. The total fluoride in the entire incisor (dentin and enamel) from the gum line to the tip is shown in Table XI. The accumulated fluoride in the incisor correlated well with fluoride exposure (Pearson correlation drinking water [F] vs. incisor [F],  $r = 0.799$ , two-tailed,  $p < 0.00001$ ), no differences were seen between the fluorosis susceptible and resistant strains and either [F] concentration. Similar body burden of fluoride, as judged from analysis of mineralized tissues, was seen in all strains despite differences in their predispositions to develop dental fluorosis.

#### QUANTITATIVE LIGHT-INDUCED FLUORESCENCE (QLF) ANALYSIS AS A QUANTITATIVE MEASURE OF FLUOROSIS

Fluorescence of teeth and its implication on caries diagnosis has been studied.<sup>136</sup>

Dental fluorosis has been observed on images during caries examinations with the QLF



technique, presenting as diffuse dark areas.<sup>127</sup> Due to the nature of fluorosis in human teeth as increased porosity in the outer layers of the enamel, there is a good possibility that it may prove useful for such purposes. Angmar-Mansson (1994)<sup>128</sup> proposed that the laser fluorescence method might be developed into a method applicable for the assessment of the severity of enamel fluorosis.

Mouse mandibular incisors were subjected to QLF analysis. One pair of mandibular central incisors from each animal was dissected, rinsed briefly in 10-percent formalin, and placed in a labeled microfuge tube. The areas of the teeth that were affected by fluorosis as judged by clinical examination showed up as bright areas on the QLF images, indicating an increase in fluorescence. The amount and area of the fluorescent signal were sufficient to discriminate groups of animals with different severities of dental fluorosis (Table XII).

We wished to determine if there was significant correlation between clinical exams where a diagnosis and staging of fluorosis was obtained. There was strong correlation between QLF and the upper incisor examinations (Pearson's Correlation, two-tailed,  $r = -0.766$ ,  $p = 0.004$ ). We show that QLF analysis provides a quantitative measurement of fluorosis. Furthermore, the difference between the most sensitive strain (A/J) and the most resistant strain (129P3/J) was large. At 50 ppm, A/J vs. 129P3/J  $p = 0.005$ , and A/J vs. CBA/J  $p = 0.006$  (Student's t-test, two-tailed, paired, where  $p < 0.05$  is considered significant).



## TABLES AND FIGURES

TABLE I

Diagnostic criteria and weighting system  
for Dean's Index<sup>b</sup>

Classification and Weight	Original Criteria (Dean, 1934) <sup>c</sup>	Modified Criteria (Dean, 1942) <sup>a</sup>
Normal 0	The enamel presents the usual translucent semi-vitriform type of structure. The surface is smooth and glossy and usually of a pale creamy white color.	The enamel presents the usual translucent semi-vitriform type of structure. The surface is smooth, glossy, and usually of a pale creamy white color.
Questionable 0.5	Slight aberrations in the translucency of normal enamel, ranging from a few white flecks to occasional white spots, 1 to 2 mm in diameter.	The enamel discloses slight aberrations from the translucency of normal enamel, ranging from a few white flecks to occasional white spots. This classification is utilized in those instances where a definite diagnosis of the mildest form of fluorosis is not warranted and a classification "normal" not justified.
Very Mild 1.0	Small, opaque, paper-white areas are scattered irregularly or streaked over the tooth surface. It is principally observed on the labial and buccal surfaces, and involves less than 25 percent of the tooth surfaces of the particular teeth affected. Small pitted white areas are frequently found on the summits of the cusps. No brown stain is present in the mottled enamel of this classification.	Small, opaque, paper-white areas scattered irregularly over the tooth but not involving as much as approximately 25 percent of the tooth surface. Frequently included in this classification are teeth showing no more than about 1-2 mm of white opacity at the tips of the summits of the cusps of the bicuspid or second molars.
Mild 2.0	The white, opaque areas on the surfaces of the teeth involve at least half of the tooth surface. The surfaces of molars, bicuspid, and cuspid subject to attrition show thin white layers worn off and the bluish shades of underlying normal enamel. Faint brown stains are sometimes apparent, generally on the upper incisors.	The white opaque areas in the enamel of the teeth are more extensive but do not involve as much as 50 percent of the tooth.

(continued)



TABLE I (continued)

Moderate 3.0	No change is observed in the form of the tooth, but generally all of the tooth surfaces are involved. Surfaces subject to attrition are definitely marked. Minute pitting is often present, generally on the labial and buccal surfaces. Brown stain is frequently a disfiguring complication. It must be remembered that the incidence of brown stain varies greatly in different endemic areas, and many cases of white opaque mottled enamel, without brown stain, are classified as "moderate" and listed in this category.	All enamel surfaces of the teeth are affected, and surfaces subject to attrition show marked wear. Brown stain is frequently a disfiguring feature.
Moderately Severe 3.5	Macroscopically, a greater depth of enamel appears to be involved. A smoky white appearance is often noted. Pitting is more frequent and generally observed on all the tooth surfaces. Brown stain, if present, is generally deeper in hue and involves more of the affected tooth surfaces.	
Severe 4.0	The hypoplasia is so marked that the form of the teeth is at times affected, the condition often being manifest in older children as a mild pathologic incisal-occlusal abrasion. The pits are deeper and often confluent. Stains are widespread and range from a chocolate brown to almost black in some cases.	Includes teeth formerly classified as "moderately severe" and "severe". All enamel surfaces are affected, and hypoplasia is so marked that the general form of the tooth may be affected. The major diagnostic sign of this classification is the discrete or confluent pitting. Brown stains are widespread, and teeth often present a corroded-like appearance.



TABLE II

Diagnostic criteria and scoring system  
for the Thylstrup and Fejerskov Index<sup>a</sup>

Score	Original Criteria (Thylstrup and Fejerskov, 1978) <sup>b</sup>	Modified Criteria (Fejerskov et al., 1988) <sup>c</sup>
0	Normal translucency of enamel remains after prolonged air-drying.	The normal translucency of the glossy, creamy-white enamel remains after wiping and drying of the surface.
1	Narrow white lines located corresponding to the perikymata.	Thin white opaque lines are seen running across the tooth surface. The lines correspond to the position of the perikymata. In some cases, a slight "snow-capping" of cusps/incisal edges may also be seen.
2	<u>Smooth surfaces</u> : More pronounced lines of opacity which follow the perikymata. Occasionally confluence of adjacent lines.	The opaque white lines are more pronounced and frequently merge to form small cloudy areas scattered over the whole surface. "Snowcapping" of incisal edges and cusp tips is common.
	<u>Occlusal surfaces</u> : Scattered areas of opacity <2 mm in diameter and pronounced opacity of cuspal ridges.	
3	<u>Smooth surfaces</u> : Merging and irregular cloudy areas of opacity. Accentuated drawing of perikymata often visible between opacities.	Merging of the white lines occurs, and cloudy areas of opacity occur spread over many parts of the surface. In between the cloudy areas, white lines can also be seen.
	<u>Occlusal surfaces</u> : Confluent areas of marked opacity. Worn areas appear almost normal but usually circumscribed by a rim of opaque enamel.	
4	<u>Smooth surfaces</u> : The entire surface exhibits marked opacity or appears chalky white. Parts of surface exposed to attrition appear less affected.	The entire surface exhibits marked opacity or appears chalky white. Parts of the surface exposed to attrition or wear may appear to be less affected.

(continued)



TABLE II (continued)

4	<u>Occlusal surfaces</u> : Entire surface exhibits marked opacity. Attrition is often pronounced shortly after eruption.	
5	<u>Smooth and occlusal surfaces</u> : Entire surface displays marked opacity with focal loss of outermost enamel (pits) <2 mm in diameter.	The entire surface is opaque, and there are round pits (focal loss of the outermost enamel) that are less than 2 mm in diameter.
6	<u>Smooth surfaces</u> : Pits are regularly arranged in horizontal bands <2 mm in vertical extension.	The small pits may frequently be seen merging in the opaque enamel to form bands that are less than 2 mm in vertical height. In this class are also included surfaces where the cuspal rim of facial enamel has been chipped off, and the vertical dimension of the resulting damage is less than 2 mm.
	<u>Occlusal surfaces</u> : Confluent areas <3mm in diameter exhibit loss of enamel. Marked attrition.	
7	<u>Smooth surfaces</u> : Loss of outermost enamel in irregular areas involving < ½ of entire surface.	There is a loss of the outermost enamel in irregular areas, and less than half the surface is so involved. The remaining intact enamel is opaque.
	<u>Occlusal surfaces</u> : Changes in the morphology caused by merging pits and marked attrition.	
8	<u>Smooth and occlusal surfaces</u> : Loss of outermost enamel involving greater than ½ of the surface.	The loss of the outermost enamel involves more than half the enamel. The remaining intact enamel is opaque.
9	<u>Smooth and occlusal surfaces</u> : Loss of main part of enamel with change in anatomical appearance of surface. Cervical rim of almost unaffected enamel is often noted.	The loss of the major part of the outer enamel results in a change of the anatomical shape of the surface/tooth. A cervical rim of opaque enamel is often noted.



TABLE III

Diagnostic criteria and scoring system for the  
Tooth Surface Index of Fluorosis (TSIF)<sup>a,b</sup>

Score	Criteria
0	Enamel shows no evidence of fluorosis.
1	Enamel shows definite evidence of fluorosis, namely, areas with parchment-white color that total less than one-third of the visible enamel surface. This category includes fluorosis confined only to incisal edges of anterior teeth and cusp tips of posterior teeth ("snowcapping").
2	Parchment-white fluorosis totals at least one-third of the visible surface, but less than two-thirds.
3	Parchment-white fluorosis totals at least two-thirds of the visible surface.
4	Enamel shows staining in conjunction with any of the preceding levels of fluorosis. Staining is defined as an area of definite discoloration that may range from light to very dark brown.
5	Discrete pitting of the enamel exists, unaccompanied by evidence of staining of intact enamel. A pit is defined as a definite physical defect in the enamel surface with a rough floor that is surrounded by a wall of intact enamel. The pitted area is usually stained or differs in color from the surrounding enamel.
6	Both discrete pitting and staining of the intact enamel exist.
7	Confluent pitting of the enamel surface exists. Large areas of enamel may be missing, and the anatomy of the tooth may be altered. Dark-brown stain is usually present.



TABLE IV

Diagnostic criteria and scoring system for  
the Fluorosis Risk Index (FRI)<sup>a,b,c</sup>

Classification and Score	Criteria
Negative 0	A surface zone will receive a score of 0 when there is absolutely no indication of fluorosis being present. There must be a complete absence of any white spots or striations, and tooth surface coloration must appear normal.
Questionable 1	Any surface zone that is questionable as to whether there is fluorosis present (i.e., white spots, striations, or fluorotic defects cover 50 percent or less of the surface zone).
Positive: Mild-to-Moderate 2	A smooth surface zone will be diagnosed as being positive for enamel fluorosis if greater than 50 percent of the zone displays parchment-white striations typical of enamel fluorosis. Incisal edges and occlusal tables will be scored as positive for enamel fluorosis if greater than 50 percent of that surface is marked by the snowcapping typical of enamel fluorosis.
Positive: Severe 3	A surface zone will be diagnosed as positive for severe fluorosis if greater than 50 percent of the zone displays pitting, staining, and deformity, indicative of severe fluorosis.
Non-fluoride opacity 7	Any surface zone that has an opacity that appears to be a non-fluoride opacity.
Excluded 9	A surface zone is categorized as excluded (i.e., not adequately visible for a diagnosis to be made) when any of the following conditions exist: incomplete eruption, orthodontic appliances or bands, crowned or restore surfaces, gross plaque, and debris.

TABLE V

Modified Developmental Defects of Enamel Index (DDE)<sup>a</sup>

<b>Modified DDE Index For Use in General Purpose Epidemiological Studies</b>		
		Code
Normal		0
Demarcated Opacities:		
White/cream		1
Yellow/brown		2
Diffuse opacities:		
Diffuse-Lines		3
Diffuse-Patchy		4
Diffuse-Confluent		5
Confluent/patchy + staining + loss of enamel		6
Hypoplasia:		
Pits		7
Missing Enamel		8
Any other defects		9
Extent of defect		
Normal		0
< 1/3		1
At least 1/3 < 2/3		2
At least 2/3		3
<b>Modified DDE Index For Use in Screening Surveys</b>		
		Code
Normal		0
Demarcated Opacity		1
Diffuse Opacity		2
Hypoplasia		3
Other Defects		4
<sup>99</sup> a		



TABLE VI  
CMYK analysis of shade guide <sup>a</sup>

Color Group	Shade Guide color code	Cyan (%)	Magenta (%)	Yellow (%)	Black (%)
3	712	23	54	96	20
3	713	29	44	95	28
3	714	33	52	92	44
3	717	34	44	87	32
2	711	27	31	82	15
2	709	32	25	75	13
2	718	36	33	73	20
2	710	35	26	68	13
1	716	22	22	58	5
1	723 <sup>b</sup>	4	3	17	0
1	724 <sup>b</sup>	0	0	1	0
1	722 <sup>b</sup>	0	0	0	0

<sup>a</sup> CMYK analysis performed by multiple imaging the shade guide under fluorescent lighting using the Nikon Coolpix 990 digital camera and processing the images using Adobe Photoshop 5.5. Colors were ranked according to percent yellow content. The separate groups were arbitrarily assigned.

<sup>b</sup> Color code created to better include white and off white shades.

TABLE VII

Observer 1 (MF) correlations

	Fluoride <sup>1</sup>	TFI 35 <sup>2</sup>	Dean 35	TFI Digital <sup>3</sup>	Dean Digital
Fluoride	1 <sup>4</sup>	.404	.392	.546	.570
TFI 35	.404	1	.855	.796	.789
Dean 35	.392	.855	1	.739	.784
TFI Digital	.546	.796	.739	1	.878
Dean Digital	.570	.789	.784	.878	1

<sup>1</sup> Fluoride treatment (0 ppm, 25 ppm, or 50 ppm as NaF in the drinking water).<sup>2</sup> 35 mm images as kodachrome slides, n = 215.<sup>3</sup> 3.2 megapixel images, n = 216.<sup>4</sup> Pearson's correlation coefficient, r, where  $p < 0.01$  for all cases.



TABLE VIII

Observer 2 (MM) correlations

	Fluoride <sup>1</sup>	TFI 35 <sup>2</sup>	Dean 35	TFI Digital <sup>3</sup>	Dean Digital
Fluoride	1 <sup>4</sup>	.354	.370	.541	.551
TFI 35	.354	1	.764	.701	.751
Dean 35	.370	.764	1	.758	.745
TFI Digital	.541	.701	.758	1	.834
Dean Digital	.551	.751	.745	.834	1

1 Fluoride treatment (0 ppm, 25 ppm, or 50 ppm as NaF in the drinking water).

2 35 mm images as kodachrome slides, n = 215.

3 3.2 megapixel images, n = 216.

4 Pearson's correlation coefficient, r, where  $p < 0.01$  for all cases.

TABLE IX

Correlation between observers 1 (MF) and 2 (MM)

TFI	TFI	Dean's	Dean's
35 mm Images <sup>1</sup>	Digital Images <sup>2</sup>	35 mm Images	Digital Images
.805 <sup>3</sup>	.989	.804	.936

<sup>1</sup> 35 mm images as kodachrome slides, n = 215.<sup>2</sup> 3.2 megapixel images, n = 216.<sup>3</sup> Pearson's correlation coefficient, r, where  $p < 0.01$  for all cases.



TABLE X  
Fluoride accumulation in femurs

Mouse Strain	[F] in Drinking Water (ppm)	Minimum [F] ( $\mu\text{g/g}$ )	Maximum [F] ( $\mu\text{g/g}$ )	Mean [F] ( $\mu\text{g/g}$ )	Standard Deviation ( $\mu\text{g/g}$ )
129P3/J	0	227.33	469.85	335.60	92.33
	25	1170.34	3065.48	2962.15	734.83
	50	3208.39	3730.51	3413.49	221.63
A/J	0	192.93	364.01	293.58	71.91
	25	1863.73	2649.69	2221.46	289.82
	50	2905.08	4559.24	3295.02	628.51
CBA/J	0	190.17	366.96	272.91	71.89
	25	571.28	1691.10	1305.39	457.85
	50	2865.78	3886.64	3376.36	423.74
SJL/J	0	96.69	263.62	202.73	73.44
	25	1737.54	2869.28	2307.03	406.16
	50	3238.31	5008.94	3867.68	683.78

One-way ANOVA was used to compare each mouse strain with another for bone [F] concentration at each treatment group. At 0 ppm and 50 ppm there was no significant difference between the strains ( $p = 0.576$  and  $0.255$ , respectively). At 25 ppm however,  $p = 0.003$ . Multiple comparisons at this treatment level using Bonferroni revealed for: A/J vs 129P3/J,  $p = 1.000$ ; A/J vs CBA/J,  $p = 0.007$ ; A/J vs SJL/J,  $p = 1.000$ ; CBA/J vs 129P3/J,  $p = 0.027$ ; and CBA/J vs SJL/J,  $p = 0.005$ .

TABLE XI

Fluoride accumulation in femurs and mandibular incisors of 129P3/J and A/J mice

Mouse Strain	[F] in Drinking Water (ppm)	Mean Incisor [F] ( $\mu\text{g/g}$ )	Standard Deviation ( $\mu\text{g/g}$ )	Mean Femur [F] ( $\mu\text{g/g}$ )	Standard Deviation ( $\mu\text{g/g}$ )
129P3/J					
	0	449.92	277.92	335.60	92.33
	25	1106.05	510.88	2962.15	734.83
	50	2047.46	332.88	3413.49	221.63
A/J					
	0	169.43	68.96	293.58	71.91
	25	1157.77	585.84	2221.46	289.82
	50	2758.22	1260.48	3295.02	628.51

One-way ANOVA was used to compare each mouse strain with another for bone [F] concentration at each treatment group. At 0 ppm and 50 ppm there was no significant difference between the strains ( $p = 0.576$  and  $0.255$ , respectively).

Pearson correlation, drinking water [F] vs. femur [F],  $r = 0.869$ , two-tailed,  $p < 0.00001$ .

Pearson correlation, drinking water [F] vs. incisor [F],  $r = 0.799$ , two-tailed,  $p < 0.00001$ .



TABLE XII

Quantitative light-induced fluorescence (QLF)  
analysis of lower incisors

Strain	[F] ppm	Delta Q (mean $\pm$ S.D.)	Student's <i>t</i> -test 0 ppm vs 50 ppm
129P3/J	0	0.07 $\pm$ 0.12	p = 0.413
	50	0.42 $\pm$ 0.92	
A/J	0	0.12 $\pm$ 0.16	p = 0.014
	50	34.35 $\pm$ 11.79	
CBA/J	0	0.13 $\pm$ 0.21	p = 0.117
	50	4.55 $\pm$ 5.63	
SJL/J	0	5.85 $\pm$ 2.05	p = 0.466
	50	8.47 $\pm$ 7.31	

Fluoride concentration present in water given *ad libitum* throughout the study period (60 days).

Images were taken using the *in vitro* QLF set-up, capturing 10 frames, inverted, and analyzed in QLF program version 2.00f, threshold was set at -3 percent.

Delta Q = [Delta F (average loss of fluorescence) x area (mm<sup>2</sup>)].

Student's *t*-test, two-tailed, paired,  $p \leq 0.05$  considered significant.

At 50 ppm, A/J vs 129P3/J  $p = 0.005$ , and A/J vs CBA/J  $p = 0.006$ .

Correlation between QLF and upper incisor exams (Pearson's Correlation, two-tailed,  $r = -0.766$ ,  $p = 0.004$ ).

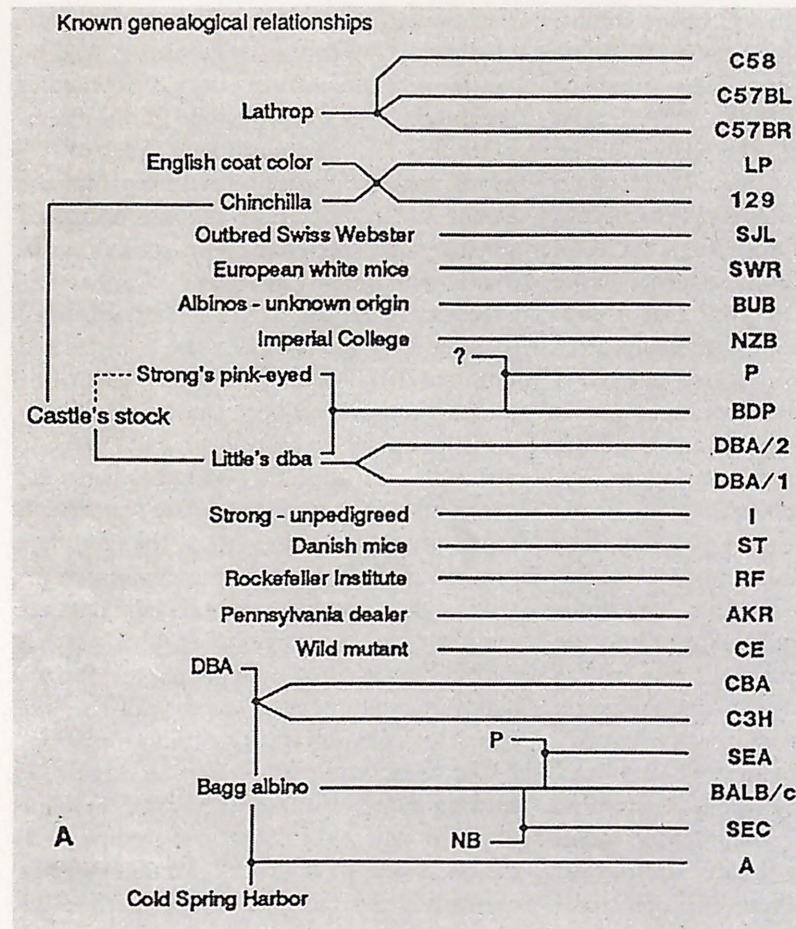


FIGURE 1. Genealogy of 24 inbred strains of mice.

The gene tree based on protein loci provides an accurate picture of the genealogical relationships among strains. (Adapted from Atchley et al.<sup>112</sup>)



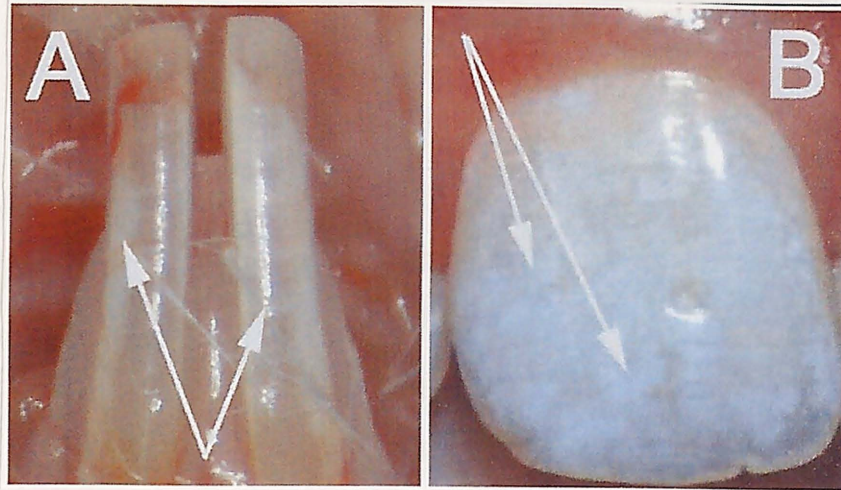


FIGURE 2: Dental fluorosis seen in an A/J mouse compared to dental fluorosis seen in an adult human patient. Panel A, lower incisors of an A/J mouse treated with 50 ppm fluoride in the drinking water for 60 days; Panel B right central incisor of an adult female demonstrating dental fluorosis (courtesy of Dr. Bruce Matis, Operative Dentistry, IUSD). Arrows point to regions of white chalky enamel, opacity, and mottling. Horizontal bands or striations are readily visible on the lower incisors of the A/J mouse.



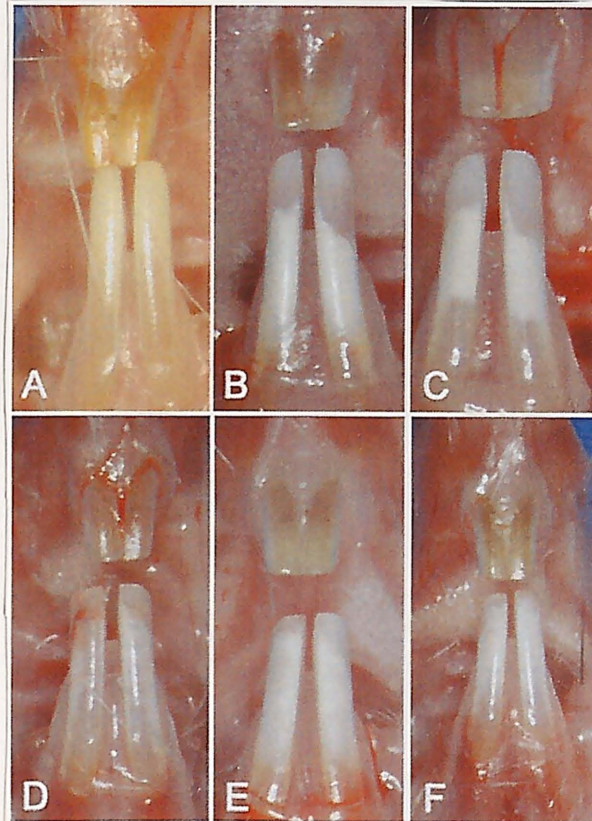


FIGURE 3. Severe dental fluorosis seen in the A/J strain. Representative A/J mice after 60 days of treatment (Panel A, 0 ppm fluoride and panels B through F, 50 ppm fluoride). Upper and lower show dramatic color and character changes in response to fluoride. Note the diffuse yellow-orange color present in the teeth of the mouse shown in Panel A, typical of normal tooth color development in A/J mice. At 0 ppm, the lower incisors are somewhat translucent. The translucency is almost completely lost at 25 ppm (not shown), and at 50 ppm (Panels B-F), little or no translucency can be appreciated over the entire tooth surface. The labial surface of the lower incisors has become rough and pitted. At sites where there is a transition (distal aspects), distinct ledges are present. Even though only 5 mice in this treatment group are shown above, all 6 animals exhibited extensive and severe fluorosis.



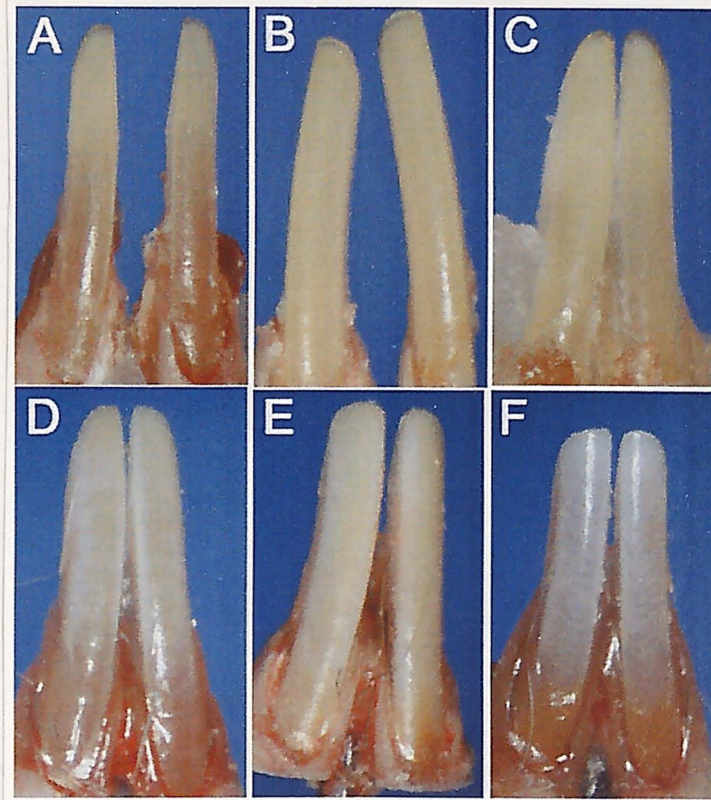


FIGURE 4. Variable dental fluorosis is seen in all strains treated. Mice at the 50 ppm concentration exhibited varying degrees of dental fluorosis depending solely on strain. 129P3/J mice (A and D) appeared to be the least affected. CBA/J (B and E) and FVB/NJ (C and F) strains exhibited mild to moderate fluorosis, respectively. Variation in the number and width of horizontal white bands present in the lower incisors of each strain above can be appreciated. Panels A, B, and C (0 ppm [F]). Panels D, E, and F (50 ppm [F]).



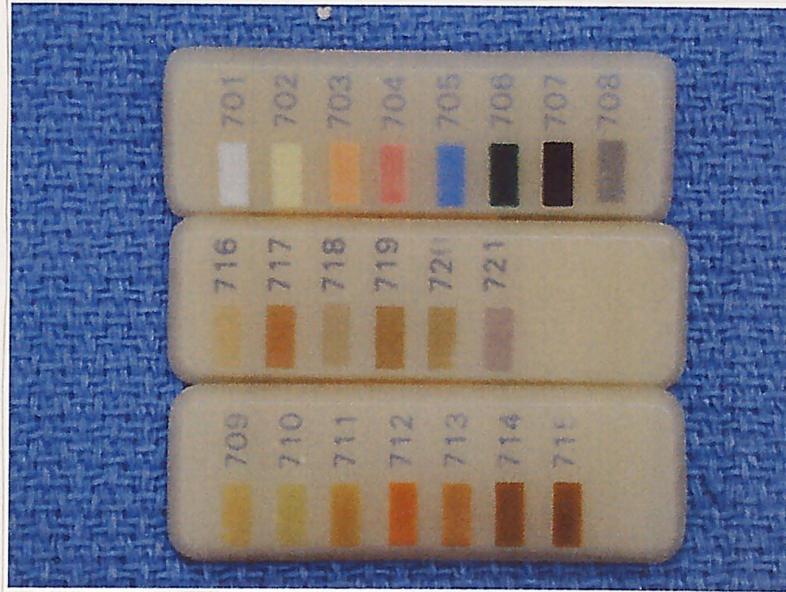


FIGURE 5. Shade guide used for incisor color determination. The shades 709, 710, 711, 712, 713, 714, 716, 717, and 718 above were seen in the mice studied. Additional shades, 723 (closely resembling the background color of the acrylic), 722, and 724 were added to better represent the various white shades seen. Ceramic custom coloring opaque powder shade guide (Vident, Brea, CA).



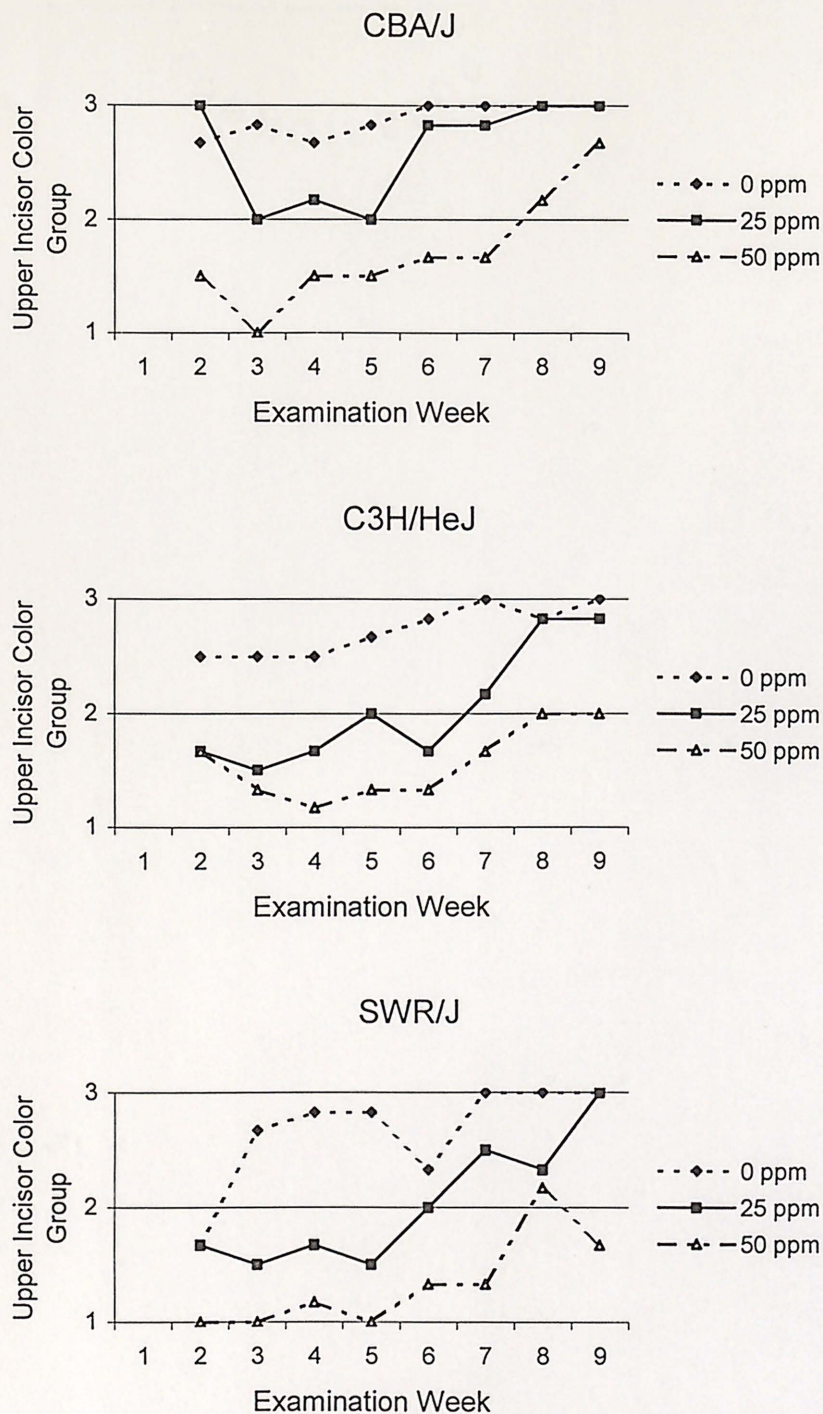


FIGURE 6a. Upper incisor color charting. The shade guide color code converted to groups 1, 2, or 3 and the average value for the 6 mice per treatment group plotted. Mouse strains responded differently to fluoride treatment. 0 ppm [F] = closed circles, 25 ppm = closed squares with dashes, and 50 ppm = triangles.

(continued)

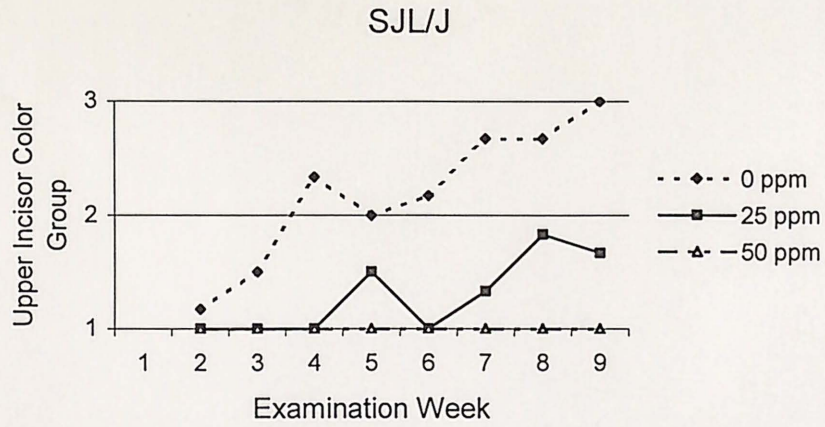


FIGURE 6a (continued). Upper incisor color charting. The shade guide color code converted to groups 1, 2, or 3 and the average value for the 6 mice per treatment group plotted. Mouse strains responded differently to fluoride treatment. 0 ppm [F] = closed circles, 25 ppm = closed squares with dashes, and 50 ppm = triangles.



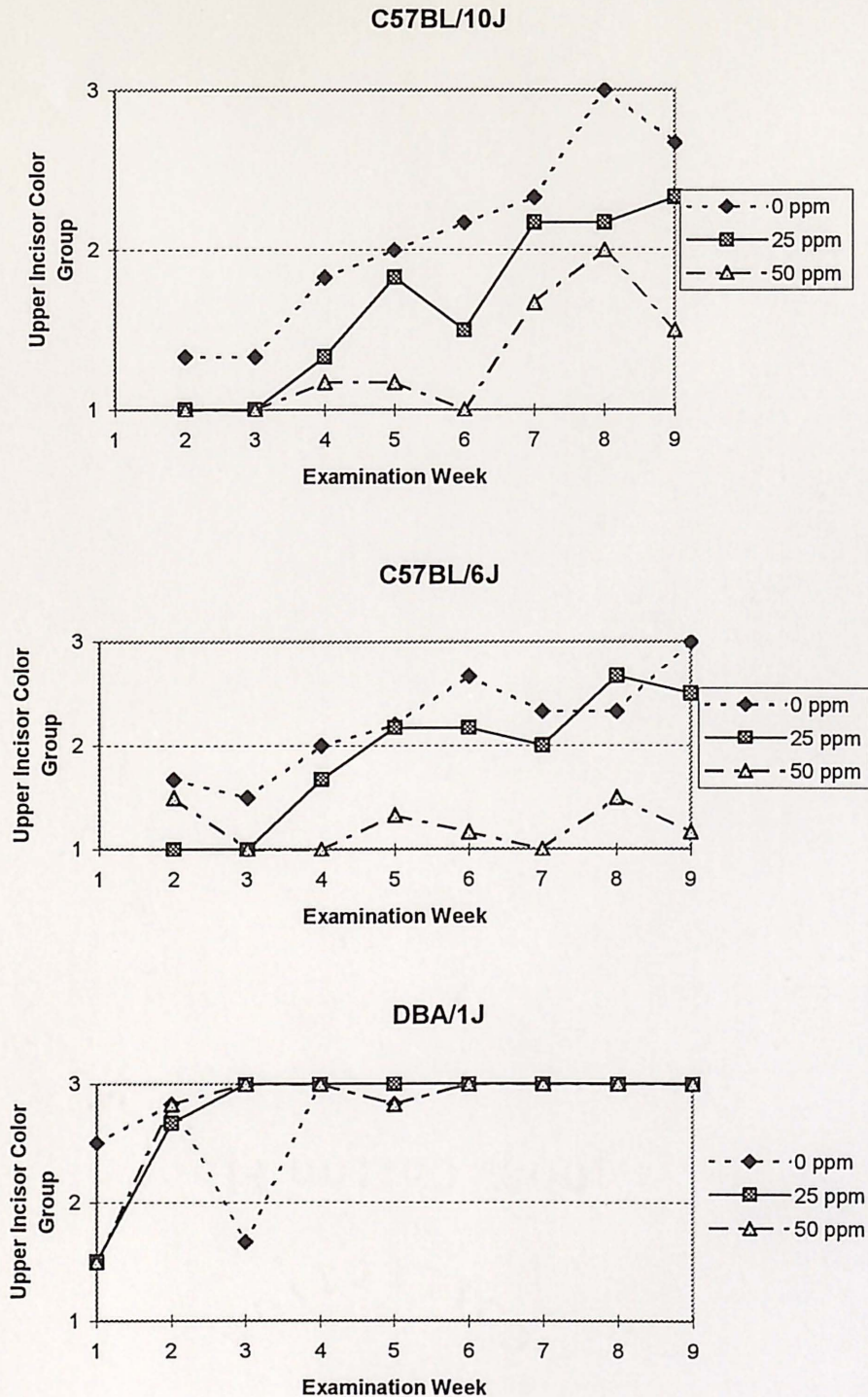


FIGURE 6b. Upper incisor color charting. The shade guide color code converted to groups 1, 2, or 3 and the average value for the 6 mice per treatment group plotted. Mouse strains responded differently to fluoride treatment. 0 ppm [F] = closed circles, 25 ppm = closed squares with dashes, and 50 ppm = triangles.

(continued)

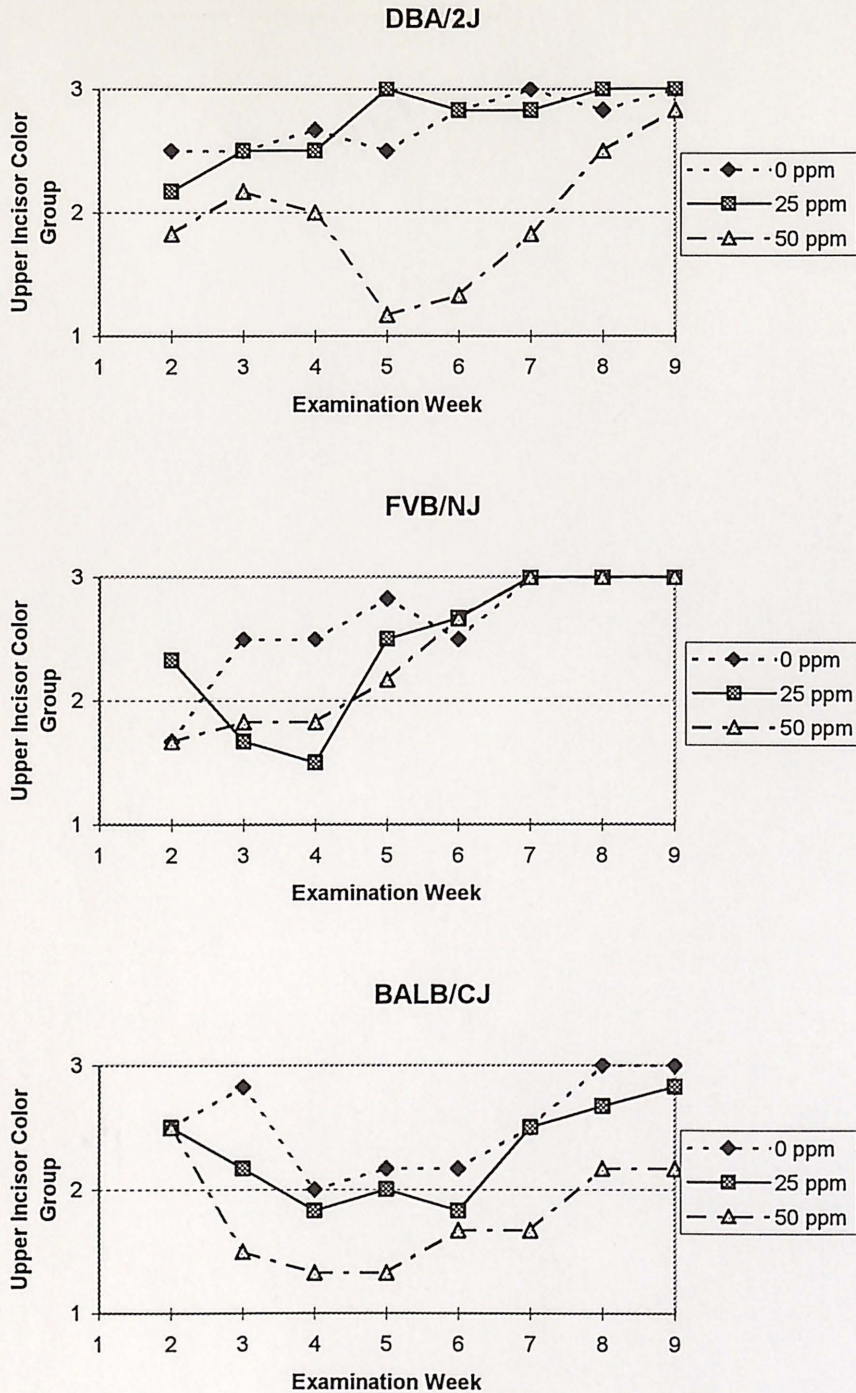


FIGURE 6b (continued). Upper incisor color charting. The shade guide color code converted to groups 1, 2, or 3 and the average value for the 6 mice per treatment group plotted. Mouse strains responded differently to fluoride treatment. 0 ppm [F] = closed circles, 25 ppm = closed squares with dashes, and 50 ppm = triangles.



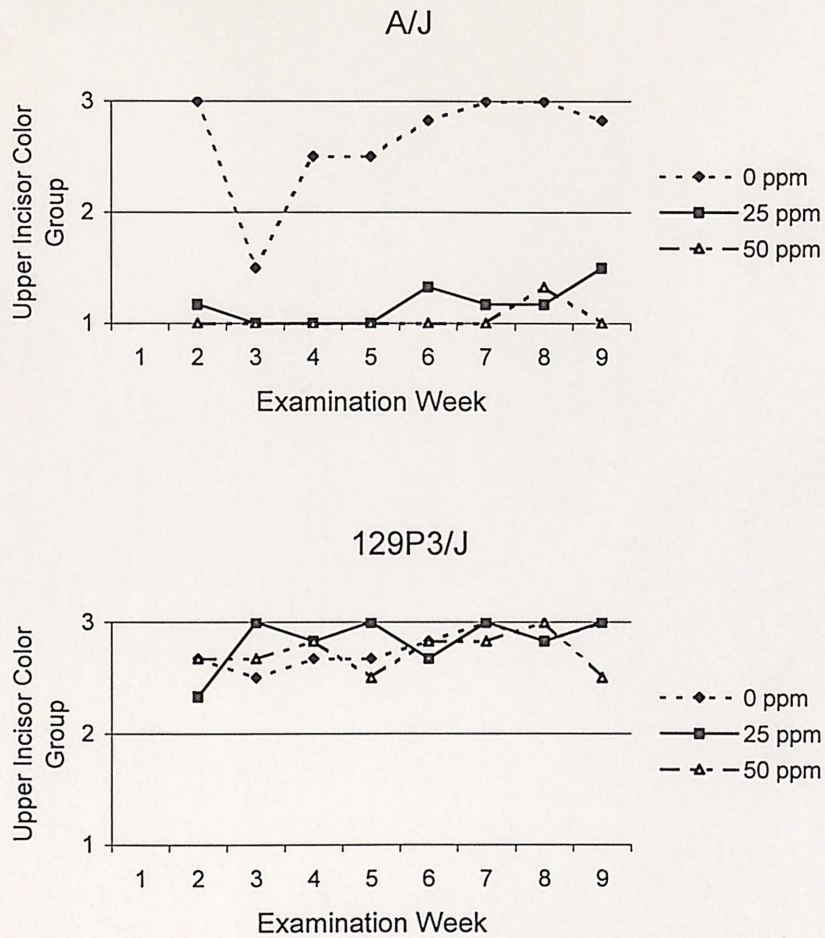


FIGURE 7. Upper incisor color charting. The shade guide color code converted to groups 1, 2, or 3 and the average value for the 6 mice per treatment group plotted. 0 ppm [F] = closed circles, 25 ppm = closed squares with dashes, and 50 ppm = triangles.

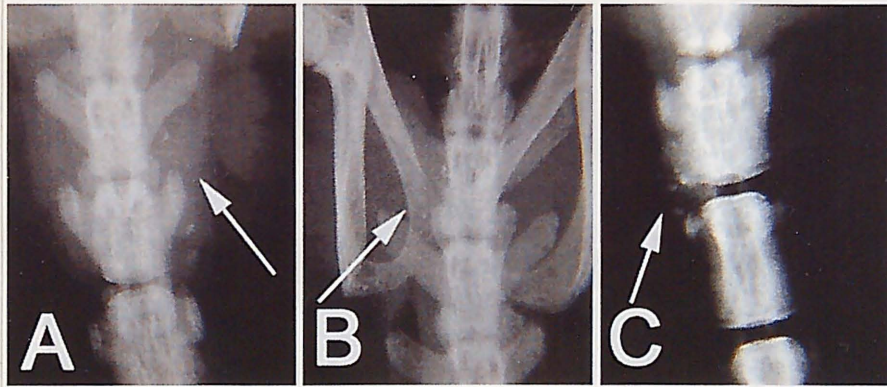


FIGURE 8. Full skeletal radiographs of SJL/J mice treated with 50 ppm F. Full skeletal surveys were performed on euthanized mice as described in the materials and methods section titled skeletal radiography. Three radiographs showed a few isolated areas of scattered calcifications, indicated by the white arrows.



## DISCUSSION

Many studies have identified the rodent as a useful model for determining the effects of fluoride on dental development (well-reviewed by Richards<sup>81</sup>). The mouse remains an important model for understanding the pathogenesis of dental fluorosis for a number of reasons. First, the continual growth of mandibular and maxillary incisors in mice permits monitoring of the effects of fluoride on new enamel formation.<sup>137</sup> Second, each inbred mouse strain represents a genetically homogenous group that often differs quite remarkably from mice in another inbred strain.<sup>112</sup> Finally, inbred mouse strains have been used for genetic studies because of the isogenicity within a strain and the genetic heterogeneity between inbred strains.<sup>110</sup>

The clinical presentation of dental fluorosis in mice paralleled that which could be seen in mild forms of dental fluorosis in humans. Variation in the onset and severity of dental fluorosis among different inbred strains of mice (when age, gender, food, housing, and fluoride exposure were rigorously controlled) supports the influence of genetic background on dental fluorosis susceptibility/resistance. The identification of dental fluorosis-susceptible and fluorosis-resistant inbred mouse strains will permit genetic dissection of the genes and pathways involved in enamel formation that underlie the pathogenesis of dental fluorosis. Few studies have been conducted that explore an underlying genetic basis for fluoride resistance. Recently, Katsura<sup>138</sup> used high concentrations of sodium fluoride to isolate fluoride-resistant mutants of *Caenorhabditis elegans*. Further study of these mutant roundworms has led to the identification of a



novel fluoride resistant gene, and an ion channel belonging to the degenerin/epithelial sodium channel superfamily that regulates defecation rhythm.<sup>138,139</sup>

At harvest, 35 mm and digital images were taken of each mouse to apply two fluorosis indices, the TF and Dean's indices, to them. These two indices were useful in this study for classifying the stage or severity of fluorosis in mice. However, the size of the mice teeth and the severity of dental fluorosis that the mice develop indicate that these indices may be more suited for human classification. With some modification, these indices show promise in their use for future mouse studies.

It is important to note that the digital images (compared with the 35 mm slide images) were more conducive to application of the fluorosis indices. This is due to the much larger size and manipulability of the images. A higher correlation was seen between observers with the digital images. It would be suggested that for future studies, digital images should be used over 35 mm slide images.

Full skeletal radiographs were taken of all mice in this project to determine if there was evidence of gross skeletal lesions or evidence of bone dysplasia. Weanling (three-week-old) male mice were chosen to assess the effects of fluoride on the skeleton well into adulthood (~13 weeks of age). From the radiographs, no gross skeletal lesions or evidence of bone dysplasia were noted. However, mild effects consisting of small ectopic calcifications around the pelvis and base of tail were present in three of the six SJL/J mice treated at  $[F] = 50$  ppm.

Perhaps if this study would have extended for a greater period of time, more evidence of skeletal fluorosis may have been noted in the mice, especially at the higher



fluoride treatment groups. There are likely other factors contributing to the development of dental fluorosis that are not completely understood.

Fluoride analyses were performed throughout this study. Analyses were performed on the deionized and fluoridated water used to treat the mice, as well as on the mouse chow fed to the mice. These analyses were performed to ensure consistency in the treatment groups, and to rigorously control food and drinking water given to the mice. It would be strongly suggested that for future studies, these rigorous controls be implemented as well.

Differences in dental fluorosis between mouse strains begged the question of whether total fluoride burden was different among the strains examined. Fluoride accumulates in mineralized tissues. Body fluoride burden was assessed, utilizing standard techniques, by determining the quantity of fluoride in femurs and mandibular incisors.

The results show that the concentration of fluoride in the incisors and femurs correlated well with fluoride concentration in the drinking water. Mice accumulated similar amounts of fluoride in their long bones regardless of whether they exhibited mild to severe dental fluorosis. The accumulated fluoride in the incisor correlated well with fluoride exposure, and no differences were seen between the susceptible and resistant strains at either fluoride concentration. The body burden of fluoride correlated well with fluoride exposure, and no statistical differences were noted between the strains.

Clear differences were seen between the A/J (sensitive) and 129P3/J (resistant) strains. One would expect to see less fluoride in the incisors and femurs of the resistant strain, but this was not the case. Similar amounts of fluoride were seen in the incisors



despite the fact that they responded so differently to fluoride and developed a much milder form of fluorosis. These results are impressive. It would be suggested that future studies include a method by which to assess body burden of fluoride. The use of femurs and incisors proved useful in this study to assess body burden of fluoride.

One disadvantage to the use of mandibular incisors is that the total fluoride of the entire incisor (dentin and enamel) from the gum line to the tip was obtained. Since the erupted mandibular incisors have enamel covering only the labial surface, and because it averages 0.01 mm thick, it is technically not possible to separate the enamel from the dentin in fluoride determinations. Much of the material measured was from the dentin. If this technical dilemma could be addressed in future studies, then perhaps the results of the fluoride analyses of the incisors would be different.

Quantitative light-induced fluorescence (QLF) was utilized in this study to detect changes in enamel, specifically to see the differences in fluorotic versus unaffected and mildly affected enamel. At harvest, one pair of mandibular incisors were removed from each animal to be subjected to QLF analysis. The areas of the teeth that were affected by fluorosis on visual examination showed up as bright areas on the QLF images, indicating an increase in fluorescence. The images were analyzed using the QLF software to quantify the average increase in fluorescence and determine the size of the affected area of the teeth.

The amount and area of the fluorescent signal were sufficient to discriminate groups of animals with different severities of dental fluorosis. The results show that there was a strong correlation between QLF results and the clinical examinations, as well as a strong correlation between QLF results and fluoride exposure. Dramatic differences were



seen between the A/J (sensitive) strain and the 129P3/J (resistant) strain. Quantitative light-induced fluorescence (QLF) has emerged in our studies as an innovative tool to detect, stage, and quantify dental fluorosis in the mouse model. The use of QLF in future mouse and human studies shows great promise.

Our investigation of fluoride burden in mineralized tissues suggests that active removal of fluoride from the body is not a sufficient explanation for dental fluorosis resistance. Our data also suggest that resistant mice are more tolerant to fluoride in their enamel organ microenvironment, whereas susceptible mice are more sensitive to the amount of fluoride within their enamel organ microenvironment.



## SUMMARY AND CONCLUSIONS

It is well-accepted that fluoride interacts with mineralized tissues and at elevated concentrations disturbs the mineralization process<sup>5</sup>, the molecular mechanisms that underlie the pathogenesis of dental fluorosis are not known. We hypothesized that genotype could influence susceptibility or resistance to develop dental fluorosis. We tested this hypothesis using a mouse model system where continuous eruption of the mouse incisor over a relatively short period of time could be monitored, and where genotype, age, gender, food, housing, and drinking water fluoride level could be rigorously controlled.

The purpose of the study was to determine if there were differences in fluoride sensitivity or resistance among different inbred mouse strains. Because mice like other rodents continue to grow their upper and lower incisors, the effects of fluoride on new enamel formation could be monitored. We were particularly interested in determining if differences in fluoride sensitivity or resistance existed among genetically disparate strains of mice.

This study involved 12 separate inbred strains of mice: 129P3/J, A/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BL/10J, CBA/J, DBA1/J, DBA/2J, FVB/NJ, SJL/J, and SWR/J. Each strain was chosen based upon maximum potential unrelatedness.<sup>110</sup> Each strain involved three groups of animals, and the study was conducted for 60 days with termination of treatment after 60 days. The three treatment groups consisted of 72 mice



each (6 each from the 12 strains studied). Group 1 received distilled water only; Group 2 received 25 ppm fluoride, and Group 3 received 50 ppm fluoride.

The deionized and prepared treatment waters were periodically analyzed by ion specific electrode (ISE) for fluoride concentration. Periodic fluoride determinations of different batches of mouse chow used in this study were performed as well.

Once a week, each mouse was given a complete oral examination (by MM and ETE) where incisor tooth color was assessed along the entire labial surface of the enamel using a shade guide (Vita Shade Guide, Vident, Brea, CA). Opacity, length (in mm using a periodontal probe), wear pattern, and overall development of the upper and lower incisors were also independently judged.

Mice were euthanized after 60 days of treatment time. During euthanasia, selected mineralized tissues were removed. Clinical materials (images and radiographs), incisor QLF, bone ash weights, and hard tissue fluoride analyses were performed on samples in numerical ascending order. Only when all data had been acquired was the code broken to permit correlation between different strains and different treatment groups.

Digital and 35 mm images of the upper and lower incisors were performed on all mice at the conclusion of treatment (at termination). The coded images were subjected to clinical assessment of fluorosis (by MM and MF) using the Dean's and modified Thylstrup and Fejerskov (TF) indices of fluorosis.

All mice were subjected to full skeletal surveys and skull radiographs from two views (dorsoventral and lateral cephalogram). The radiographs were an important



component in the evaluation of skeletal fluorosis. We investigated any gross skeletal changes using standardized radiography.

At harvest, one pair of mandibular incisors from each animal were dissected away from the hemi-mandibles for QLF analysis. The findings and conclusions of this study can be seen summarized below.

1. Examination of 12 genealogically disparate inbred strains of mice showed differences in dental fluorosis susceptibility/resistance between the strains. Furthermore, we found clustering of strains into distinct phenotypic groups.
2. Clinical fluorosis seen in mice parallels fluorosis in humans. When fluorosis developed, the upper and lower incisors appeared white, chalky, and opaque. Striations and erosions of the enamel were evident and particularly prominent on the lower incisors.
3. Longitudinal study of upper incisor color permitted mice to be grouped into three phenotypic groups: resistant strains (129P3/J, FVB/NJ, CBA/J, and DBA/1J); intermediate strains (SWR/J, BALBc/J, C57BL/10J, and DBA/2J); and sensitive strains (A/J, SJL/J, C3H/HeJ, and C57BL/6J).
4. The A/J inbred mouse strain is highly susceptible, with a rapid onset and severe development of dental fluorosis compared with the other strains tested. Whereas, the 129P3/J mouse strain is least affected with minimal dental fluorosis.
5. The above observations directly support the contribution of a genetic component in the pathogenesis of dental fluorosis.



6. Both the TF and the Dean's indices are useful for classifying the stage or severity of fluorosis in mice. There are advantages to the use of digital images over conventional 35 mm slide images.
7. Both the TF and the Dean's indices correlate well with the amount of fluoride exposure during amelogenesis. However, these indices are not promising indicators of the fluoride burden during amelogenesis.
8. From the skeletal radiography, no gross skeletal lesions or evidence of bone dysplasia were noted. However, mild effects consisting of small ectopic calcifications around the pelvis and base of tail were present in three of the six SJL/J mice treated at  $[F] = 50$  ppm.
9. Pearson's Correlation, two-tailed, was significant between fluoride concentration in water and fluoride concentration in the femur,  $r = 0.869$ ,  $p < 0.00001$ . A dose response also was noted between fluoride in the water and fluoride in the bone. Overall, mice accumulated similar amounts of fluoride in their long bones regardless of whether they exhibited mild to severe dental fluorosis. Small but significant differences were appreciated at 25 ppm.
10. The accumulated fluoride in the incisor correlated well with fluoride exposure (Pearson's Correlation drinking water  $[F]$  vs. incisor  $[F]$ ,  $r = 0.799$ , two-tailed,  $p < 0.00001$ ), no differences were seen between the fluorosis-susceptible and fluorosis-resistant strains and either F concentration. Similar body burden of fluoride, as judged from analysis of mineralized tissues, was seen in all strains despite differences in their predispositions to develop dental fluorosis.

11. The innovative use of quantitative light induced fluorescence (QLF) has emerged as a tool to quantify dental fluorosis in the mouse model.
12. There was strong correlation between QLF and the upper incisor examinations (Pearson's Correlation, two-tailed,  $r = -0.766$ ,  $p = 0.004$ ).



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## ABSTRACT

GENETIC SUSCEPTIBILITY/RESISTANCE TO FLUOROSIS  
AMONG DIFFERENT MOUSE STRAINS

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Fluoridation of community water supplies for the purpose of preventing dental caries remains one of the top 10 public health interventions of the last century. However, exposure (ingestion) of greater than optimal amounts of fluoride from a variety of sources has led to an increase in the prevalence of dental fluorosis. We propose that dental fluorosis represents a complex condition caused by environmental and genetic factors.

Purpose: To assess the role of genetics in the pathogenesis of dental fluorosis using genetically separate inbred strains of mice. Methods: Twelve genealogically disparate strains of mice were treated with 0 ppm, 25 ppm, and 50 ppm of fluoride in their drinking water. Each mouse was given weekly dental fluorosis evaluations. After 60 days of treatment, femurs were collected for fluoride analysis. Mandibular incisors were isolated for quantitative light induced fluorescence (QLF) studies and fluoride analysis. Digital and 35 mm images were taken of all mouse incisors in order to apply and compare the Dean's Index and the modified Thylstrup and Fejerskov Index (TFI), both indices of dental fluorosis. Skeletal radiographs were taken on the euthanized mice and



later examined for extra skeletal calcifications and other gross bony deformities. Results: Differences in dental fluorosis susceptibility/resistance were identified between the strains, ranging from mild, moderate, to severe dental fluorosis. Furthermore, we found clustering of strains into distinct phenotypic groups. The A/J mouse strain was highly susceptible, with a rapid onset and severe development of dental fluorosis compared with the other strains tested. The 129P3/J mouse strain was least affected with negligible dental fluorosis. From the skeletal radiographs, no gross skeletal lesions or evidence of bone dysplasia were noted. Similar body burden of fluoride, as judged from analysis of mineralized tissues, was seen in all strains despite differences in their predispositions to develop dental fluorosis. Both the Dean's and TF indices are useful for classifying the stage or severity of fluorosis in mice, and there are advantages to the use of digital images over conventional 35 mm slide images. Both indices correlate well with the amount of fluoride exposure during amelogenesis; however, these indices are not promising indicators of fluoride burden during amelogenesis. Conclusions: QLF proved to be an innovative and useful tool for the quantification of dental fluorosis. Furthermore, these observations support the role of a genetic component in the pathogenesis of fluorosis.

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